

# THE PROKARYOTES

Third Edition

A Handbook on the Biology of Bacteria:  
Proteobacteria: Gamma Subclass

*Edited by*

MARTIN DWORKIN (EDITOR-IN-CHIEF)


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ERKO STACKEBRANDT

Volume 6

 Springer

Third Edition

# **A Handbook on the Biology of Bacteria**

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**Volume 6: Proteobacteria: Gamma Subclass**

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# Preface

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

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

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

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

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

*Martin Dworkin*  
Editor-in-Chief

# Foreword

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

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

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

well as much of the philosophy may be traced to the Delft School, passed on to us by our teachers, Martinus Beijerinck, A. J. Kluyver, and C. B. van Niel, and in turn passed on by us to our students.

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

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

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

To prepare a treatise such as *The Prokaryotes* requires dedicated editors and authors; the task has been enormous. I predict that the scientific community of microbiologists will again show its appreciation through use of these volumes—such that the pages will become “dog-eared” and worn as students seek basic information for the

hunt. These volumes belong in the laboratory, not in the library. I believe that a most effective way to introduce students to microbiology is for them to isolate microbes from nature, i.e., from their habitats in soil, water, clinical specimens, or plants. *The Prokaryotes* enormously simplifies this process and should encourage the construction of courses that contain a wide spectrum of diverse topics. For the student as well as the advanced investigator these volumes should generate excitement.

Happy hunting!

Ralph S. Wolfe  
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## Proteobacteria

## Gamma Subclass

## New Members of the Family Enterobacteriaceae

J. MICHAEL JANDA

In the last edition of this chapter, Don Brenner (Brenner, 1991) wrote:

“They are, nonetheless, important for a variety of reasons. They have innate importance as members of the family, to be compared and contrasted with their relatives, in attempts to define the phenotypic, phylogenetic, and ecological boundaries of the family.”

No words better define the current edition of this chapter as the number of genera of Enterobacteriaceae has risen 60%, from 15 to 24 (excluding *Raoultella*). This current list of 24 genera does not even include such organisms as *Alterococcus agarolyticus* and *Saccharobacter fermentatus*, taxa that are not formally discussed in this chapter, although they possess some characteristics associated with the family Enterobacteriaceae (Yaping et al., 1990; Shieh and Jean, 1998).

The exponential increase in new genera within the family Enterobacteriaceae has been fueled by molecular biology, including polymerase chain reaction (PCR) technology and 16S (small subunit) rDNA sequencing. This list of genera will no doubt continue to increase. What has resulted from this molecular onslaught is the basic fundamental knowledge that the family Enterobacteriaceae plays a critical role not only in enteric disease of humans and animals, but also as phytopathogens, as insect pathogens, and in industrial processes. Members of the family Enterobacteriaceae can be broadly classified into four main categories for convenience, although a number of these genera clearly overlap into multiple categories (Table 1). Probably the greatest explosion in our knowledge concerning enterobacteria stems from new insight into their role as endosymbionts of insects or of the parasites that feed on insect larvae (Moran and Baumann, 2000). Some genera, such as *Photorhabdus* and *Xenorhabdus*, may serve as new delivery vehicles of important bacterial toxins with insecticidal activity, replacing such classic agents as *Bacillus thuringiensis* (French-Constant and Bowen, 1999). Knowledge of the association that genera, such as *Brenneria* and *Pectobacterium*, have with plant diseases contin-

ues to expand as our understanding of their correct taxonomic position becomes apparent. Phylogenetic analysis has also revealed that some agents, such as *Calymmatobacterium granulomatis*, the causative agent of the sexually transmitted disease, donovanosis (granuloma inguinale), are actually members of the Enterobacteriaceae. Thus, our knowledge regarding the Enterobacteriaceae and factors regulating molecular evolution of endosymbionts, disease associations in humans, animals, and plants, biological applications, and ecologic niches will continue to expand at a rapid rate for the foreseeable future.

### *Arsenophonus* (*Androcidium*)

In 1986, Werren et al. isolated the causative agent (*Arsenophonus nasoniae*) of the son-killer (*sk*) trait from infected tissues of the parasitic wasp, *Nasonia vitropennis*. The *sk* trait, a sex-ratio distorter, is carried by approximately 5% of female wasps from natural populations (Gherna et al., 1991). Infected wasps transmit the bacterium to fly pupae (wasp host) during oviposition and offspring then become infected perorally (Werren et al., 1986). The bacterium causes the death of only male wasp eggs (unfertilized) by arresting their maturation. Werren et al. (1986) recovered the slow-growing bacterium from tryptic soy and brain heart infusion broth cultures containing either infected *sk*-wasp tissues or hemolymph of fly pupae parasitized by *sk* females. *Arsenophonus nasoniae* was subsequently proven to be the causative agent of the *sk* trait by fulfilling Koch's postulates.

*Arsenophonus nasoniae* DNA was 0–5% related to DNA from 54 representative species of Enterobacteriaceae (Brenner, 1991). However, total DNA from strain *A. nasoniae* SK14 hybridized to an rRNA *Escherichia coli* sequence enriched for, but not exclusive to, members of the family Enterobacteriaceae (Gherna et al., 1991). SK14 was 62% related at 60°C and 42% related at 70°C to the *Escherichia coli* probe. These values are similar to those

Table 1. New members of the family Enterobacteriaceae: major associations.

Human pathogens	Phytopathogens	Insect pathogens, symbionts, and endosymbionts	Environmental, industrial, and animals
<i>Calymmatobacterium</i>	<i>Brenneria</i>	<i>Arsenophonus</i>	<i>Budvicia</i>
<i>Cedecea</i>	<i>Pantoea</i> <sup>b</sup>	<i>Buchnera</i>	<i>Buttiauxella</i>
<i>Ewingella</i>	<i>Pectobacterium</i>	<i>Photorhabdus</i>	<i>Obseumbacterium</i>
<i>Kluyvera</i>		<i>Sodalis</i>	<i>Pragia</i>
<i>Leclercia</i>		<i>Wigglesworthia</i>	<i>Trabulsiella</i> <sup>c</sup>
<i>Leminorella</i>		<i>Xenorhabdus</i>	
<i>Moellerella</i> <sup>a</sup>			
<i>Rahnella</i>			
<i>Tatumella</i>			
<i>Yokenella</i> <sup>a</sup>			

<sup>a</sup>Primarily isolated from humans, but not definitely shown to be a human pathogen.

<sup>b</sup>Also causes infrequent infections in humans (e.g., *P. agglomerans*).

<sup>c</sup>Primarily an environmental group, but occasionally recovered from clinical specimens.

exhibited by other members of the Enterobacteriaceae including *Providencia*, *Proteus mirabilis* and *Xenorhabdus nematophilus*. A phylogenetic tree based upon rRNA sequences of selected members of the family Enterobacteriaceae was constructed and indicates that the genus *Proteus* is the closest neighbor to strain SKI4 (Gherna et al., 1991). This relationship is further supported by the fact that the G+C content (39.5 mol%) and fatty acid composition (large amounts of C<sub>16:0</sub> and 16:1 *cis* 9, smaller amounts of 14:0) of strain SKI4 are very similar to that of the genera *Proteus* and *Providencia*, respectively. A second putative species in the genus *Arsenophonus* has been detected as a “secondary (S)-endosymbiont” in the reduviid bug, *Triatoma infestans* (Hypša and Dale, 1997). A bacterial culture TII1 was isolated from the hemolymph of *T. infestans*. The TII1 bacteria could be propagated in vitro by cocultivation with a mosquito cell line (*Aedes albopictus*) but could not be cultured in vitro. PCR amplification of a 16S rDNA product (1361 bp) from TII1-*A. albopictus* cultures and comparison to other 16S rDNA sequences from eubacteria indicated that the endosymbiont was a member of the  $\gamma$ -3 subdivision of the class Proteobacteria (Hypša and Dale, 1997). The TII1 rDNA sequences are 96.2% related to those of *A. nasoniae* and a phylogenetic tree indicates that *A. nasoniae* is the most closely related taxa to TII1. Because TII1 cannot be cultured in vitro and valid biochemical tests cannot be performed, *Candidatus* status (provisional assignment) has been applied prior to the vernacular epithet (Murray and Schleifer, 1994; Hypša and Dale, 1997). Whether *Arsenophonus* contains the enterobacterial common antigen (ECA) is unknown.

*Arsenophonus nasoniae* is a fastidious Gram-negative, oxidase-negative, catalase-positive, non-sporeforming, nonmotile rod that produces

acid from D-glucose, fructose, and sucrose (Brenner, 1991; Gherna et al., 1991). It is unusual (with respect to the Enterobacteriaceae) in its inability to grow on MacConkey agar and to reduce nitrates. It grows optimally at 30°C, with a range from 15 to 35°C. A description of biochemical characteristics associated with *A. nasoniae* can be found in Gherna et al. (1991). The optimal pH range for growth is 7.2–7.8, with a pH range from 6.2 to 8.7 (Brenner, 1991). Limited antibiotic susceptibility studies indicate that *A. nasoniae* is resistant to polymyxin, penicillin, methicillin and erythromycin and is sensitive to gentamicin, chloramphenicol, tetracycline and sulfathiazole (Werren et al., 1986). Growth does not occur in minimal salts plus glucose without the addition of protein digests; amino acid supplements do not support growth. Vitamins, trace metals, and IsoVitalex also do not support growth (Brenner, 1991). The addition of 1% proteose peptone (Difco no. 0120) to conventional media, however, has been shown to improve growth (Gherna et al., 1991). “*Candidatus Arsenophonus triatominarum*” is also Gram negative, nonmotile, and non-sporeforming (Hypša and Dale, 1997). The rods are highly filamentous (>15  $\mu$ m in length, 1–1.5  $\mu$ m in diameter).

Both *A. nasoniae* and *Candidatus Arsenophonus triatominarum* are maternally transmitted, and both are found in the central nervous system (brain and neural ganglia) of infected insects. *Candidatus Arsenophonus triatominarum* is additionally found in the hemolymph, heart, salivary glands, visceral muscles, nephrocytes, ovaries, testes and dorsal vessels of *T. infestans* (Hypša and Dale, 1997). A sex-ratio distorter trait due to.

*Candidatus Arsenophonus triatominarum* has not been found in *T. infestans*.

The type strain of *A. nasoniae* is SKI4 (= ATCC 49151).



## *Brenneria*

The genus *Brenneria* is composed of several necrogenic phytopathogenic species that formerly resided within the “amylovora group” of the genus *Erwinia*. Phylogenetic investigations conducted on 16 *Erwinia* species utilizing 16S rDNA sequence data identified four distinct clusters, one of which (cluster IV) contained the species *E. salicis*, *E. nigrifluens* and *E. rubrifaciens* (Kwon et al., 1997). Hauben et al. (1998a) analyzed the 16S rDNA sequences of 29 plant-associated strains and also identified four clusters. In this later study, cluster III contained the three species previously found to reside in cluster IV by Kwon et al. (1997), in addition to *E. alni*, *E. paradisiaca* and *E. quercina*. These six species shared sequence similarities ranging from 94.7–97.4%. The mean sequence relatedness of cluster IV members to the genera *Erwinia* and *Pectobacterium* were 94.5 and 95.4%, respectively (Hauben et al., 1998a). Signature sequences identified among cluster III members were conserved in all six species; these sequences differed from those of cluster I (*Erwinia*), cluster II (*Pectobacterium*) and cluster IV (*Pantoea*). Based upon these results, Hauben et al. (1998a) proposed the transfer of these six *Erwinia* species to a new genus, *Brenneria*, named in honor of the American bacteriologist Don J. Brenner. Subsequent studies investigating sequence divergence in the glyceraldehyde-3-phosphate dehydrogenase (*gapDH*) gene of the glycolytic pathway support the genetic differentiation of the genera *Brenneria* and *Erwinia* (Brown et al., 2000). However, other phylogenetic studies have not demonstrated such a robust separation between *Brenneria* and *Pectobacterium* species (Spröer et al., 1999). At the DNA level, *E. salicis*, *E. nigrifluens* and *E. rubrifaciens* are 27–32% related to *E. quercina* EQ 102; *E. rubifaciens*, *E. nigrifluens* and *E. quercina* are 33–60% related to *E. salicis* ATCC 15712 at 60°C (Brenner et al., 1974). Similar relatedness values have been reported using *E. nigrifluens* (29–47%) and *E. rubrifaciens* (25–

54%) as labeled DNA. The G+C content of members of the genus *Brenneria* range from 50.1 to 56.1 mol% (Hauben et al., 1998a).

The genus *Brenneria* consists of Gram-negative, oxidase-negative, catalase-positive fermentative rods, 1.3–3.0  $\mu\text{m}$  in length by 0.5–1.0  $\mu\text{m}$  in width (Hauben et al., 1998a). Biochemically, *Brenneria* species are similar to *Pantoea*, *Pectobacterium* and *Erwinia* in that they fail to produce arginine dihydrolase and to decarboxylate amino acids such as ornithine and lysine (Lelliott and Dickey, 1984). Acid is produced from the fermentation of D-glucose, D-fructose, salicin, mannose and sucrose. Amylases are not produced. *Brenneria rubrifaciens* produces a pink diffusible pigment on YDC medium (1% yeast extract, 1% D-glucose, 2% precipitated chalk, and 2% agar). Most species are sensitive to carbenicillin, cephalothin, chloramphenicol, naladixic acid and tetracycline, and resistant to bacitracin, erythromycin and gentamicin. Differential characteristics useful in separating members of the genus *Brenneria* are listed in Table 2.

As former members of the *Erwinia* “amylovora group,” *Brenneria* species cause dry necrosis or wilt in the specific host plants they infect. *Brenneria alni* causes necrotic cankers in the bark of the trunk, branches and twigs of the Italian alder (*Alnus cordata*) and black alder (Surico et al., 1996). In the warmer months, a watery liquid is expressed from small cracks in the canker. A similar disease called “drippy nut disease” is caused by *B. quercina* in oaks. *Brenneria nigrifluens* causes bark canker in the Persian (English) walnut, while *B. rubrifaciens* causes necrosis in the Persian walnut (*Juglans regia*). *Brenneria salicis* is the causative agent of “watermark disease” in willows (*Salix* sp.). This vascular wilting disease is characterized by the appearance of wilted, dried, brown-colored leaves (Hauben et al., 1998b). Infection primarily resides within the xylem vessels of infected trees.

The type strains of *Brenneria* species are listed in Table 3.

Table 2. Distinguishing features of *Brenneria* species.

Characteristic	<i>B. alni</i>	<i>B. nigrifluens</i>	<i>B. paradisiaca</i>	<i>B. quercina</i>	<i>B. rubrifaciens</i>	<i>B. salicis</i>
Indole	–	–	+	–	–	–
$\beta$ -Galactosidase	–	+	+	+	+	+
Pectate degradation	–	–	+	–	+	+
Acid from						
L-Arabinose	+	+	+	–	+	–
Raffinose	–	+	+	–	–	+
Xylose	+	+	+	–	–	–

Based on data from Lelliott and Dickey (1984), Surico et al. (1996), and Hauben et al. (1998a).

Table 3. Type strains of *Brenneria* species.

Species	Type strain	Other designations
<i>B. alni</i>	PVFi 20	ICMP 12481; NCPPB 3934; ATCC700181
<i>B. nigrifluens</i>	EN 101(WC 1)	ICMP 1578; LMG 2694; NCPPB 564; ATCC 13028
<i>B. paradisiaca</i>	NCPPB 2511	LMG 2542; ATCC 33242
<i>B. quercina</i>	ICPB EQ 101	1; ICMP 1845; LMG 2724; NCPPB 1852; PDDCC 1845; ATCC 29281
<i>B. rubrifaciens</i>	ICPB ER 103	ICMP 1915; LMG 2709; NCPPB 2020; PDDCC 1915; ATCC 29291
<i>B. salicis</i>	NCPPB 47	BS 1027; D. Dye EX2; ICMP 1587; LMG 2698; ATCC 15712

## *Buchnera*

The genus *Buchnera* consists of a single species, *B. aphidicola*, which is an obligate intracellular symbiont of the greenbug aphid, *Schizaphis graminum* (Munson et al., 1991). *Buchnera aphidicola* cannot be cultured in vitro. The bacterium exists within 60–80 huge specialized aphid cells called “bacteriocytes” or “mycetocytes” located within host-derived membrane vesicles termed “symbiosomes” (Baumann et al., 1995). *Buchnera* is vertically transmitted to eggs and offspring by a process that is not completely understood, thereby perpetuating the association between bacterium and insect. *Buchnera* or *Buchnera*-like endosymbionts also have been reportedly found in a number of aphid species including *Rhopalosiphum maidis*, *Rhopalosiphum padi*, *Myzus persicae*, *Uroleucon sonchi*, *Acyrtosiphon pisum*, *Diuraphis noxia*, *Chaitophorus viminalis*, *Mindarus victoria*, *Pemphigus betae* and *Melaphis rhois* (Munson et al., 1991); some species of the tribe Cerataphidini are devoid of *Buchnera* endosymbionts (Baumann et al., 1995).

Based upon 16S RNA sequence data, *Buchnera* belongs to the  $\gamma$ -3 subdivision of the class Proteobacteria and is most closely related to members of the family Enterobacteriaceae (Munson et al., 1991; Baumann et al., 1995). The G+C content of *Buchnera* DNA is 30 mol%. *Buchnera aphidicola* appears to have developed a symbiotic association with a progenitor aphid species approximately 150–200 million years ago (Buades et al., 1999). The genome (630–643 kb) is only about one-seventh the size of *E. coli* (Wernegreen et al., 2000). However, the size of *Buchnera* genomes recovered from different aphid species is highly conserved (<3% difference), suggesting genome shrinkage early in the evolutionary symbiotic process. Multiple copies of the genome (>120) exist within bacteriocytes (Komaki and Ishikawa, 1999). This copy number varies depending upon the developmental stage of the aphid, increasing from post-embryonic development (0.2 times;  $10^6$  endosymbionts) to adulthood ( $5.6 \cdot 10^6$  endosymbionts) and subsequently declining with age (Baumann et al., 1998; Komaki and Ishikawa, 2000).

*Buchnera aphidicola* consists of round to oval cells (2–5  $\mu$ m in diameter) that have the general morphology of a Gram-negative cell wall lacking a flagellum (Munson et al., 1991). There is both ultrastructural and chemical evidence of the presence of peptidoglycan between two unit membranes (Baumann et al., 1995) but polyamines, with the exception of spermidine, are missing (Nakabachi and Ishikawa, 2000).

The complete genome of *Buchnera* strain APS (640, 681 bp) from the pea aphid, *Acyrtosiphon pisum*, has recently been sequenced (Shigenobu et al., 2000). A total of 583 open-reading frames (ORFs) were identified. The chromosome contains genes for the amino acid biosynthesis (histidine, serine and branched-chain families), fatty acid biosynthesis and polyamine biosynthesis (Shigenobu et al., 2000). However, it either lacks or is deficient in genes required for the synthesis of cell-surface components, including lipopolysaccharides and phospholipids, necessitating their symbiotic association with aphids (Shigenobu et al., 2000). Other *Buchnera* genes detected include those involved in DNA replication, energy metabolism, cell division, chaperons, colicin-related functions and protein translation and modification (Baumann et al., 1998; Shigenobu et al., 2000). An integrated view of *Buchnera* metabolism can be found in Shigenobu et al. (2000). Another unusual feature of the *Buchnera* genome is the presence of a single copy of the genes (16S, 23S-5S) coding for rRNA (Baumann et al., 1998). The presence of a single ribosomal gene copy is indicative of slow-growing organisms. Acetone preservation appears to be the method of choice for preserving *Buchnera* DNA in preparation for molecular analysis (Fukatsu, 1999).

Aphids primarily feed on plant phloem sap (Baumann et al., 1995). This diet is rich in carbohydrates but deficient in nitrogenous compounds. Aphids cannot synthesize 10 essential amino acids for growth (Baumann et al., 1998). Such nutrients (e.g., tryptophan and leucine) are provided by *Buchnera* endosymbionts (Baumann et al., 1995). *Buchnera* endosymbionts furnish these essential nutrients by plasmid-mediated amplification of key genes involved in tryptophan and leucine biosynthesis (Van Ham

et al., 2000). If aphids are treated with antibiotics to eliminate endosymbionts, they lose the ability to reproduce, and die prematurely (Baumann et al., 1998). Thus, the symbiotic association between aphids and *Buchnera* is essential for life.

Under Rule 18a of the *International Code of Nomenclature of Bacteria*, when a species cannot be maintained in culture, a description may serve as the type strain. On this basis, the type strain has been designated as a primary endosymbiont of *Schizaphis graminum* (Munson et al., 1991).

## *Budvicia*

The genus *Budvicia*, with its single species *B. aquatica*, was first isolated and described by Aldová and colleagues in Czechoslovakia in 1983 (Brenner, 1991). DNA relatedness studies on these strains and a formal genus and species proposal were subsequently reported collaboratively by the Czech group and a group at the Pasteur Institute in Paris (Bouvet et al., 1985; Brenner, 1991). Phylogenetic analysis of the type strain of *B. aquatica* indicates that *Budvicia* forms a distinct lineage in the family, well separated from other Enterobacteriaceae genera (Spröer et al., 1999). Sources from which *B. aquatica* has been isolated include fresh water sources (wells, water pipes, swimming pools, brooks, streams and rivers), sewage water samples, and from one shrew in Spain (Brenner, 1991; Schubert and Groeger-Söhn, 1998).

Relatedness of the other 59 strains to the type strain of *B. aquatica* was 71–100% (87% average) in 60°C DNA hybridization reactions, and divergence in related sequences was 0.0–0.2% (Brenner, 1991). Several *Budvicia*-like strains were only 9–22% related to the *B. aquatica* hybridization strain 23227HG26. Relatedness of *B. aquatica* to other Enterobacteriaceae was 8% or less. The G+C content is 46 mol% (Bouvet et al., 1985). *Budvicia aquatica* expresses the ECA (Böttger et al., 1987).

A medium has been designed for the recovery of *B. aquatica* from environmental water samples (Schubert and Groeger-Söhn, 1998).

Medium of Schubert and Groeger-Söhn (1998) for the Recovery of *B. aquatica*

Trypticase peptone	15.0 g
Soybean peptone	5.0 g
Sodium chloride	5.0 g
Sodium thiosulfate (5H <sub>2</sub> O)	10.0 g
Ferric ammonium citrate	2.0 g
Sodium deoxycholate	1.5 g
Agar	15.0 g

Dissolve ingredients in 1 liter of deionized water and adjust pH to 7.3.

To the basal medium, 7.0 µg/ml of cephalothin (sodium salt) is added. Hydrogen sulfide (H<sub>2</sub>S)-positive colonies are picked and identified as *B. aquatica* biochemically. In a study of water samples taken from rivers, streams and wastewater, *B. aquatica* was detected in low numbers (range 0.3–1.3 colony forming units (cfu)/ml) in all surface water samples analyzed (Schubert and Groeger-Söhn, 1998). An inflow water sample from a wastewater plant yielded *B. aquatica* at a concentration of 1250 cfu/ml.

*Budvicia aquatica* grows on MacConkey agar and nutrient agar at 30 and 37°C, respectively, but no growth occurs at 42°C (Brenner, 1991). At 24 h on nutrient agar, *B. aquatica* colonies are small in size (~0.1 mm) increasing to 1.5 mm in diameter after 48 h incubation at 37°C. The bacterium is motile at 22°C with variable motility at 37°C (Aldová et al., 1984). A distinguishing feature of *B. aquatica* is the production of H<sub>2</sub>S, and it must be separated from other H<sub>2</sub>S-producing members of the family Enterobacteriaceae (Table 4). Other phenotypic properties of the genus *Budvicia* include hydrolysis of urea and production of the enzyme β-galactosidase (the presence of which is indicated by hydrolysis of ONPG, *o*-nitrophenyl-β-D-galactopyranoside). Little or no gas is produced from the fermentation of carbohydrates. A detailed list of pheno-

Table 4. Differential properties of H<sub>2</sub>S-positive members of the Enterobacteriaceae.

Test	<i>Budvicia</i>	<i>Citrobacter</i>	<i>E. tarda</i>	<i>Leminorella</i>	<i>Pragia</i>	<i>Proteus</i>	<i>Salmonella</i>	<i>Trabulsiella</i>
Indole	–	V	+	–	–	–	–	V
KCN	–	+	–	–	–	+	–	+
PDA	–	–	–	–	V	+	–	+
LDC	–	–	+	–	–	–	+	+
ODC	–	V	–	–	–	V	V	V
ADH	–	V	+	–	–	–	+	+
Acid from								
L-Rhamnose	+	+	–	–	–	–	V	+
D-Xylose	+	+	–	V	–	+	V	+

Symbols: +, ≥90% positive; V, 10–90% positive, and –, ≤10% positive.

Abbreviations: KCN, growth in potassium cyanide broth; PDA, phenylalanine deaminase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; and ADH, arginine dihydrolase.

Adapted from Janda and Abbott (1998).

typic properties can be found in Bouvet et al. (1985).

The type strain of *B. aquatica* is 20186HG01 (= ATCC 25567).

## *Buttiauxella*

The genus *Buttiauxella* consists of seven named species whose primary ecologic niche appears to be as inhabitants of mollusks (Müller et al., 1996). The genus was initially defined by Ferragut and others in 1981 when a taxon previously designated “group F” was formerly proposed as a new genus, *Buttiauxella*, with a single species, *B. agrestis*. However, from the early onset it was clear that additional unrecognized species within the genus existed. Over a decade later, the genus was amended to include six additional species based upon the analysis of over 200 snail and slug strains (Müller et al., 1996). A number of these new species had been previously recognized by the Centers for Disease Control and Prevention (CDC) as separate taxa within the family Enterobacteriaceae and had been given the vernacular names “Enteric Groups 59, 63 and 64.”

The DNA relatedness of strains residing within each *Buttiauxella* species ranges from 68 to 100% at 60°C (Müller et al., 1996). Interspecies DNA-DNA relatedness values varied from 48 to 70% with highest similarity values noted between *B. agrestis* and *B. izardii* and between *B. gaviniae* (Enteric Group 64) and *B. noackiae* (Enteric Group 59). DNA relatedness between *Buttiauxella* and more than 50 other species of Enterobacteriaceae varied from 15 to 30% (Müller et al., 1996). Sequencing of 16S rDNA from all seven species indicates a high degree of phylogenetic relatedness (99.1–99.7%) with much lower similarity values (>97%) recorded between *Buttiauxella* and other genera in the family (Spröer et al., 1999). The G+C content of *Buttiauxella* is 55–57 mol% (Gavini et al., 1983b). *Buttiauxella agrestis* expresses the ECA (Böttger et al., 1987).

The primary habitat of most *Buttiauxella* species appears to be mollusks including slugs and snails. *Buttiauxella* has also been recovered from drinking water, soil and earthworms (Müller et al., 1996). One species (*B. noackiae*, Enteric Group 59) has been recovered from clinical (respiratory tract and wound) specimens and from food (Farmer et al., 1985); however, there is no evidence that *B. noackiae* was involved in clinical disease in any of the patients whose samples yielded this bacterium.

*Buttiauxella* grows well on ordinary plating media over a 4–37°C range (Janda and Abbott, 1998). Optimal growth occurs between 30 and 36°C. On nutrient agar, colonies are 1–2 mm in

diameter at 24 h and 2–3 mm in size at 48 h (Müller et al., 1996). Poor to moderate growth occurs at 42°C (*B. brennerae* fails to grow at 42°C). Phenotypically, the genus *Buttiauxella* most closely resembles *Kluyvera* (Brenner, 1991; Janda and Abbott, 1998). These two genera are most easily distinguished from one another by a combination of tests listed in Table 5.

*Buttiauxella* species are H<sub>2</sub>S-negative and separation from other H<sub>2</sub>S-negative members of the Enterobacteriaceae that inhabit aquatic ecosystems (such as *Budvicia aquatica* [20% are H<sub>2</sub>S-negative], *Enterobacter amnigenus*, *Enterobacter intermedium*, *Klebsiella planticola*, *Klebsiella terrigena*, *Rahnella aquatilis* and *Serratia fonticola*) can be problematic. Diagnostic keys to separate these genera and species can be found in Janda and Abbott (1998). Tests useful in the identification of *Buttiauxella* species can be found in Table 6. It should be remembered that in identifying *Buttiauxella* isolates to species level, a battery of biochemical tests is needed. This is due to the fact that no single biochemical test is differential for certain pairs of *Buttiauxella* species, such as *B. agrestis* and *B. izardii*.

In addition to the properties described in Table 6, there are several other noteworthy phenotypic properties of *Buttiauxella* species. Strains of *B. noackiae* segregate into two distinct phenotypes. All snail isolates have been found to be lactose-negative, while 7 of 8 human isolates (Enteric Group 59) are lactose-positive (Müller et al., 1996). *Buttiauxella warmboldiae* strains are initially *m*-inositol positive (Table 6); however, they lose their ability to ferment this non-carbohydrate compound upon prolonged storage (years), suggesting that the fermentation of this compound is not constitutively expressed. Limited susceptibility studies have been performed on *B. agrestis* (Freny et al., 1988). The results of testing 13 *B. agrestis* strains by broth microdilution indicates that the species is resistant to ampicillin, amoxicillin-clavulanic acid, ticarcillin, cephalothin, cefoxitin, cefotaxime, doxycycline, trimethoprim and chloramphenicol, based upon MIC<sub>90</sub> values. MIC<sub>50</sub> data indicates that many

Table 5. Biochemical properties useful in separating *Buttiauxella* and *Kluyvera* species.

Test	<i>Buttiauxella</i>	<i>Kluyvera</i>
Indole	– <sup>a</sup>	+ <sup>b</sup>
Arginine dihydrolase	V	–
Sucrose fermentation	–	V
α-Methyl-D-glucoside fermentation	– <sup>c</sup>	+

Symbols: see footnote in Table 4.

<sup>a</sup>Except *B. noackiae* (V).

<sup>b</sup>Except *K. cochleae*.

<sup>c</sup>Except *B. ferragutiae* (V) and *B. noackiae* (V).

Data from Farmer (1999).

Table 6. Identification of *Buttiauxella* species.

Test	<i>B. agestis</i>	<i>B. brennerae</i>	<i>B. ferragutiae</i>	<i>B. gaviniae</i>	<i>B. izardii</i>	<i>B. noackiae</i>	<i>B. warmboldiae</i>
Citrate	+	–	–	V	–	V	V
LDC	–	–	+	–	–	–	–
ODC	+	V	V	–	+	–	–
PDA	–	–	–	–	–	+	+
Acid from							
Adonitol	–	V	–	+	–	–	–
<i>m</i> -Inositol	–	–	–	–	–	–	V
Melibiose	+	+	–	–	V	–	–
Raffinose	+	+	–	–	V	–	–
D-Sorbitol	–	–	+	–	–	–	–

Symbols: see footnote in Table 4.

Abbreviations: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; and PDA, phenylalanine deaminase.

Data from Farmer (1999).

Table 7. Type strains of *Buttiauxella* species.

Species	Type strain	Other designations
<i>B. agrestis</i>	CUETM 77-167	ATCC 33320
<i>B. brennerae</i>	S1/6-571	ATCC 51605; DSM 9396; serial no. 145
<i>B. ferragutiae</i>	CDC 1180-81	CUETM 78-31; ATCC 51602; DSM 9390; serial no. 054
<i>B. gaviniae</i>	S1/1-984	ATCC 51604; DSM 9393; serial no. 062
<i>B. izardii</i>	S3/2-161	ATCC 51606; DSM 9397; serial no. 151
<i>B. noackiae</i>	NSW 11	ATCC 51607; DSM 9401; serial no. 170
<i>B. warmboldiae</i>	NSW 326	ATCC 51608; DSM 9404; serial no. 182

isolates, however, are susceptible to the above agents with the exception of ticarcillin, cephalothin and trimethoprim. *Buttiauxella agrestis* strains were universally susceptible to the aminoglycosides (gentamicin, tobramycin and amikacin).

The type strains of *Buttiauxella* species are listed in Table 7.

## *Calymmatobacterium*

In 1913, Aragón and Vianna cultured pleomorphic bacteria from ulcerative donovanosis (granuloma inguinale) lesions and named the isolated bacterium “*Calymmatobacterium granulomatis*” (also referred to as “*Donovania granulomatis*”). These cultured bacteria were probably not the causative agent of the disease, and no extant type strain of the species exists (Dienst and Brownell, 1984; Carter et al., 1999a); however, the name has persisted and appears on the *Approved Lists of Bacterial Names* (Skerman et al., 1980). *Calymmatobacterium granulomatis* cannot be cultured in vitro using standard microbiologic techniques and has only been isolated on rare occasions using specialized assays. Amplified genomic DNA from *C. granulomatis* extracted from tissue biopsy specimens or from coculture with monocytes hybridizes with universal Gram-negative but not Gram-positive probes (Kharsany et al., 1999). 16S rDNA sequencing

indicates that *C. granulomatis* contains signature sequences present in members of the  $\gamma$  subclass of Proteobacteria. DNA sequences encoding the *phoE* gene (344 bp) have been amplified from ulcerative material from three patients with the clinical diagnosis of donovanosis. These *phoE* sequences are most closely related to *Klebsiella* species, in particular, *K. ozaenae* (0.3% divergence), *K. pneumoniae* (0.6% divergence), *K. rhinoscleromatis* (1.16% divergence) and *K. oxytoca* (7.8% divergence); they are less related (>25.8% divergence) to other enterobacteria (Bastian and Bowden, 1996). Other studies support close nucleotide similarities (99.7–99.8%) between the *phoE* genes (1001 bp) of *C. granulomatis*, *K. rhinoscleromatis* and *K. pneumoniae* (Carter et al., 1999a). 16S rRNA sequences (1088 bp) are also highly related between these three species (98.8–99.8%), although one study (Kharsany et al., 1999) found lower 16S rDNA similarity values between *C. granulomatis* and *K. pneumoniae* (95%) and *Enterobacter* (94%) than those described by Carter et al. (1999a). Based upon the cited phylogenetic data and the similarity in disease presentation between *C. granulomatis* and *K. rhinoscleromatis*, the proposal has been made to transfer *C. granulomatis* to the genus *Klebsiella* as *K. granulomatis* comb. nov. (Carter et al., 1999a). Because of inability to perform DNA-DNA hybridization studies, the mol% G+C content of *C. granulomatis* is not available.

Humans are the only known reservoir for *C. granulomatis*. Although *C. granulomatis* was reputedly isolated from both embryonated eggs and in cell-free culture medium in studies conducted between 1943 and 1962, recent investigations have failed to confirm these earlier observations. *Calymmatobacterium granulomatis* has been grown in vitro by cocultivation of amikacin-treated tissue biopsy specimens from genital ulcers of patients with a clinical picture of granuloma inguinale with peripheral blood monocytes (Kharsany et al., 1996). These studies have been expanded by other investigators using either HEp-2 cells or monocytes (Carter et al., 1997; Kharsany et al., 1997). Often antimicrobial agents such as cycloheximide, vancomycin, benzylpenicillin and metronidazole are added to prevent the growth in coculture of contaminant microorganisms that are present in tissue biopsy specimens.

*Calymmatobacterium granulomatis* is a highly pleomorphic Gram-negative rod (Dienst and Brownell, 1984). Morphologic varieties of the bacterium can range from immature forms ovoid to elliptical in shape (0.6–1.0  $\mu\text{m}$  in length), to mature curved-to rod-shaped bacteria 0.5–1.5  $\mu\text{m}$  in width to 1.0–2.0  $\mu\text{m}$  in length. Extracellular bacteria grown in cocultures yield bacilli with bulging or tapered ends exhibiting single or bipolar staining with Giemsa (Kharsany et al., 1997). Giemsa staining also reveals a halo surrounding mature bacilli indicating the presence of a capsule. Encapsulation has been confirmed by ultrastructural investigations. Transmission electron micrographs of *C. granulomatis* bacilli indicate a typical Gram-negative cell wall with an outer membrane, a middle opaque layer, and an inner plasma membrane (Kharsany et al., 1997). Although *C. granulomatis* is a Gram-negative bacterium, it stains poorly with Gram reagents.

Identification of *C. granulomatis* in genital ulcers has been traditionally dependent upon demonstrating the presence of Donovan bodies in infected mononuclear cells or histiocytes (Richens, 1991). Donovan bodies can be visualized using a number of staining techniques including Wright's stain and Giemsa stain. They typically appear as pleomorphic bodies (see above) with bipolar staining giving a closed "safety-pin" appearance. The sensitivity of the technique is 60–80% depending upon the stage of the disease and the type of smear or specimen analyzed (Richens, 1991). Donovan bodies must be distinguished from other intracellular bodies or inclusions, such as rhinoscleroma, leishmaniasis, lymphogranuloma venerum, histoplasmosis, chronic lymphocytic cervicitis and malakoplakia (Richens, 1991). Coculture provides a second alternative method for detecting and visualizing intracellular and extracellular *C. granulomatis*

(Kharsany et al., 1996; Carter et al., 1997; Kharsany et al., 1997). Recently, PCR probes have been developed to detect *C. granulomatis* using swabs rather than biopsy/tissue smears (Carter et al., 1999b). The specificity of the assay is based upon two unique base changes in the *phoE* gene of *C. granulomatis*, which eliminates *Hae*III restriction sites and results in a 167-bp PCR product that is present in *C. granulomatis* but not closely related to the corresponding DNA segment in *Klebsiella* species (Carter et al., 1999b). A colorimetric adaptation of this PCR assay has been developed for routine use in diagnostic laboratories (Carter and Kemp, 2000). All 14 specimens from persons with donovanosis gave strong colorimetric readings while eight unrelated genital specimens were negative.

Donovanosis (granuloma inguinale) is a chronic ulcerative disease of the genitalia and is most commonly seen in tropical climates such as is found in southeast India, the Caribbean, South America, regions of Africa, and in southern China (Richens, 1991; Hart and Rao, 1999). It is thought that *Calymmatobacterium granulomatis* is sexually transmitted. The typical incubation period ranges from 3 to 90 days (1–6 weeks). Four major patterns of donovanosis presentation and disease progression have been identified (Hart and Rao, 1999). These include 1) ulcerative or ulcero-granulomatous disease (the most common pattern), 2) the necrotic or phagedenic type seen in persons with long-standing donovanosis, 3) the hypertrophied type, and rarely, 4) the sclerotic type. Although the genitalia are the most common sites of infection, distal sites may also become infected, such as the neck and mouth (Richens, 1991). Despite the fact that susceptibility studies on *C. granulomatis* cannot be performed, the disease responds to a wide variety of antimicrobial agents, including tetracycline compounds, macrolides, chloramphenicol, and co-trimoxazole (Richens, 1991; Hart and Rao, 1999).

No type strain of *C. granulomatis* currently exists.

## *Cedecea*

The genus *Cedecea* contains three named and two unnamed species, all of which were formerly placed in Enteric Group 15 on the basis of their positive lipase reaction and resistance to colistin and cephalothin (Grimont et al., 1981; Brenner, 1991). DNA relatedness studies revealed five hybridization groups in the 17 strains originally tested. Nine strains were *Cedecea davisae*, five were *Cedecea lapagei*, and one each represented unnamed *Cedecea* species (Brenner, 1991). In the original publication, the unnamed species

were called *Cedecea* species “001,” “002,” and “012.” Subsequently, *Cedecea* species 001 was referred to as “*Cedecea* species 3,” *Cedecea* species 002 as “*Cedecea* species 4,” and *Cedecea* species 012 as “*Cedecea* species 5” (Farmer, 1984; Farmer et al., 1985; Brenner, 1991). A second strain of *Cedecea* species 4 was isolated from blood cultures of a patient with possible endocarditis, and *Cedecea* species 4 was then named “*Cedecea neteri*” (Farmer et al., 1982). A strain possibly representing a sixth *Cedecea* species was isolated from ulcers on the right heel and the left ankle of a 79-year-old man with a history of diabetic arthritis of both legs (Hansen and Glupczynski, 1984). The G+C content is 49–50 mol% for *C. davisae*, 48–52 mol% for *C. lapagei*, and unknown for *C. neteri* (Brenner, 1991). All nomenclatures and *Cedecea* species 3 and 5 produce the ECA (Ramia et al., 1982; Böttger et al., 1987).

Originally, all *Cedecea* isolates had been recovered from humans (Brenner, 1991). However, *Cedecea lapagei* (n = 1) and a *Cedecea* species (n = 1) were subsequently isolated from laboratory-reared oriental fruit flies, *Dacus dorsalis* (Jang and Nishijima, 1990). One strain of *C. davisae* has also been isolated from imported vegetables (Österblad et al., 1999). In a study of *Cedecea* strains received by the CDC, over 70% of the 34 culture-collection isolates were recovered from either the respiratory tract or wounds (Farmer et al., 1985). Other less common anatomic sites yielding *Cedecea* species included blood, stool, urine, gall bladder and eye (Brenner, 1991). The medical significance of *Cedecea* is difficult to assess since most isolates have been recovered from polymicrobial processes and from persons that are severely immunocompromised. *Cedecea davisae*, the most commonly isolated *Cedecea* species, has been isolated from the greatest variety of anatomical specimens (Brenner, 1991; Janda and Abbott, 1998). Bae and Sureka (1983) reported on a scrotal abscess infection in a 50-year-old man with alcoholic hepatitis caused by *C. davisae*. Drainage and culture yielded heavy growth of *C. davisae* and only rare *Staphylococcus epidermidis* colonies. *Cedecea davisae* has also been associated with bacteremia in a 70-year-old man with heart and chronic obstructive pulmonary disease (Perkins et al., 1986). In this case, the role of *C. davisae* is less clear since the patient initially suffered from *S. aureus* bacteremia upon admission (day 4) and was only culture-positive for *C. davisae* from blood, central venous pressure catheter, and Swan-Ganz tips at day 26 of hospitalization. *Staphylococcus epidermidis* was also cultured from the same catheter tips and blood further clouding the issue. An interesting sidelight is the fact that light growth of *C. davisae* was also obtained on

successive days from sputum cultures on this patient during his septic episode, although the clinical picture did not suggest pneumonia. *Cedecea lapagei* has been exclusively recovered from respiratory tract specimens (throat, sputum and lung); for five of these isolates, no clinical information is available (Grimont et al., 1981). A sixth isolate has been recovered from a 60-year-old male with pulmonary disease and a past history of pulmonary tuberculosis and a cavitory lesion consistent with a fungus ball (Coudron and Markowitz, 1987). Although *C. lapagei* was isolated from two lung tissue specimens (pure culture), bronchial washings, and two sputum samples, an etiologic role in the patient’s respiratory problems could not be established. Probably the best evidence for a pathogenic role for *Cedecea* in human disease stems from two case reports concerning *C. neteri* infection. Farmer et al. (1982) described a case of *C. neteri* bacteremia in a 62-year-old man with heart disease and possible endocarditis. Three sets of blood cultures (taken at different times) were positive for *C. neteri*. More recently, a fatal case of *C. neteri* bacteremia has been reported in a 27-year-old woman with systemic lupus erythematosus (Aguilera et al., 1995). In this case report, three sets of blood cultures were positive for *C. neteri* during a septic crisis that the patient experienced 36 days after admission to the hospital. This nosocomial infection was thought to have been involved in the fulminant outcome leading to the patient’s death on the 42<sup>nd</sup> day of hospitalization. *Cedecea* species 3 has been isolated from post-mortem heart blood of a 76-year-old man who suffered from ethanol dependency, chronic and acute pancreatitis, and micronodular cirrhosis of the liver (Mangum and Radisch, 1982). *Candida albicans* sepsis was the apparent cause of death. No significance to the blood isolate of *Cedecea* species 3 could be ascribed. *Cedecea* species 3 has also been isolated from a wound and the respiratory tract (Farmer et al., 1985). The only isolate of *Cedecea* species 5 was from a wound.

*Cedecea* superficially resemble *Serratia* in their ability to elaborate lipase and their resistance to colistin and cephalothin (Brenner, 1991). *Cedecea* can be separated from other members of the Enterobacteriaceae by negative tests for lysine decarboxylase, gelatinase, and deoxyribonuclease, their inability to ferment L-arabinose and L-rhamnose and a positive arginine dihydrolase test (Janda and Abbott, 1998). The gelatinase and deoxyribonuclease reactions clearly separate *Cedecea* from *Serratia* (Janda and Abbott, 1998). Differential tests used to identify *Cedecea* species can be found in [Table 8](#).

Certain strains of *Cedecea* emit a distinctive “potato-like” odor, a trait shared with some

Table 8. Differential tests for *Cedecea* species.

Test	<i>C. davisae</i>	<i>C. lapagei</i>	<i>C. neteri</i>	Species 3	Species 5	Species 6 (?)
Acetate	–	V	–	V	V	+
Malonate	+	+	+	–	–	+
ODC	+	–	–	–	V	–
Acid from						
Lactose	V	V	V	–	–	–
Melibiose	–	–	–	+	+	ND
Raffinose	–	–	–	+	+	ND
Sucrose	+	–	+	V	+	+
D-Sorbitol	–	–	+	–	+	+
D-Xylose	+	–	+	+	+	–

Symbols: see footnote in Table 4; and ND, not determined.

Abbreviation: ODC, ornithine decarboxylase.

Data from Brenner (1991).

Table 9. Type strains of *Cedecea* species.

Species	Type strain	Other designations
<i>C. davisae</i>	005	CDC 3278-77; CIP 80.34; ATCC 33431
<i>C. lapagei</i>	004	CDC 0485-76; CIP 80.35; ATCC 33432
<i>C. neteri</i>	002	CDC 0621-75; ATCC 33855

strains of *Serratia*. The potato-like odor is due to a complex mixture of pyrazines (Janda and Abbott, 1998). No formal studies have been published on the antimicrobial susceptibility of *Cedecea* species. What little information is available comes from individual case reports. Most strains appear to be resistant to both ampicillin and first-generation cephalosporins and susceptible to trimethoprim-sulfamethoxazole, tetracycline, ticarcillin and the aminoglycosides (Janda and Abbott, 1998). Susceptibility to second- and third-generation cephalosporins was variable.

The type strains of *Cedecea* species are listed in Table 9.

## *Ewingella*

Enteric Group 40 contained 10 strains from human clinical specimens that were thought to resemble a lipase-negative *Cedecea* biogroup (Brenner, 1991). DNA relatedness showed that these strains constituted a single new species (Grimont et al., 1983). At 60°C, nine of these strains were 86 ± 10% related to the type strain, CDC 1468-78. The new species was 21% or less related to other Enterobacteriaceae (closest species *Rahnella aquatilis*) by the S1 nuclease-trichloroacetic acid (TCA) method. Enteric Group 40 was placed in a new genus *Ewingella* as *Ewingella americana*. The change in melting temperature ( $\Delta T_m$ ) values between eight *E. americana* strains and strain CDC 1468-78

ranged from 1.2 to 2.5°C (Grimont et al., 1983). However, a ninth strain (CDC 0679-79) exhibited a  $\Delta T_m$  value of 5.5°C, suggesting that a possible subspecies within *E. americana* may exist. The G+C content is 54 mol% (Grimont et al., 1983). *Ewingella americana* produces the ECA (Böttger et al., 1987).

The ecologic niches that *E. americana* inhabits have dramatically expanded over the past decade. Farmer et al. (1985) described a CDC collection of 44 strains, most of which originated from respiratory sources (n = 18), blood (n = 11) or wounds (n = 7). All strains were isolated from humans, with the exception of one food isolate. Pumpuni and others (Pumpuni et al., 1993) have since reported the isolation of *E. americana* from the intestinal tract of a laboratory colony of mosquitoes (*Anopheles stephensi*). A study of 282 mollusks by Müller et al. (1995) resulted in the recovery of 23 strains of *E. americana* from slugs (primarily) and snails. However, since most of the mollusks were not found to harbor *E. americana*, it does not appear to be part of the normal intestinal flora of slugs and snails. Thirty-five strains of *E. americana* have also been isolated from the cultivated mushroom, *Agaricus bisporus* (Inglis and Peberdy, 1996). One mushroom strain (P198) was found to be 88.8% related to the type strain of *E. americana* using a DNA filter hybridization technique.

*Ewingella americana* is a peritrichously flagellated Gram-negative rod (1.0–1.8  $\mu$ m in length by 0.6–0.7  $\mu$ m in width) with characteristics typical of other members of the Enterobacteriaceae. Growth occurs between 15–37°C but not at 40°C (Grimont et al., 1983). *Ewingella americana* grows in peptone water containing 8% NaCl at 30°C. Although *E. americana* was initially thought to be phenotypically similar to a lipase-negative biogroup of *Cedecea*, it can be distinguished from the latter group in that *Ewingella* is malonate-negative, fails to produce gas from glucose fermentation, and is DL-lactate-



Table 10. Differential properties of unusual LDC-, ODC-, ADH-, and H<sub>2</sub>S-negative Enterobacteriaceae.

Test	<i>Ewingella americana</i>	<i>Pantoea spp.</i>	<i>Leclercia</i>	<i>Moellerella</i>	<i>Photorhabdus/Xenorhabdus</i>	<i>Rahnella</i>	<i>Tatumella</i>
Motility	V	V	+	-	+	-	-
Indole	-	V	+	-	V	-	-
PDA	-	V	-	-	-	+	+
VP	+	V	-	-	-	+	-
Acid from L-Arabinose	-	+	+	-	-	+	-

Symbols: see footnote in Table 4.

Abbreviations: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; ADH, arginine dihydrolase; H<sub>2</sub>S, hydrogen sulfide; PDA, phenylalanine deaminase; and VP, Voges-Proskauer.

Data from Janda and Abbott (1998) and Farmer (1999).

negative, while *Cedecea* is positive for all of these traits. However, *Ewingella* needs to be separated from other genera within the Enterobacteriaceae that are H<sub>2</sub>S-negative, lysine decarboxylase- and ornithine decarboxylase-negative, and arginine dihydrolase-negative (Table 10).

In the original publication of Grimont et al. (1983), two biotypes of *E. americana* were described. Biogroup 1 strains, which contained nine isolates, were negative for acid production from L-rhamnose, D-sorbitol and D-xylose and were  $\beta$ -xylosidase-negative. Biogroup 2, which contained only a single strain (CDC 0679-79), was positive for all of these characteristics. Since this publication, no additional strains of biogroup 2 have been isolated, although strains have been recovered that are positive for one or more of the above characters but not all four (Devreese et al., 1992). Another interesting observation is the fact that strains of *E. americana* initially isolated from mollusks and mushrooms have been lipase-positive on Tween 80 medium (Müller et al., 1995; Inglis and Peberly, 1996). In one of these studies, lipase activity was lost upon subculture suggesting that the enzyme is not constitutive (Müller et al., 1995). Chitinolytic activity has been detected in the type strain of *E. americana* and in other strains isolated from mushrooms (Inglis and Peberdy, 1996). Nitrate-negative strains of *E. americana* are also known to occur (McNeil et al., 1987a). Some strains of *E. americana* contain from one to four plasmids ranging in molecular mass from ~3 to 190 MDa (McNeil et al., 1987a). Freney et al. (1988) studied the susceptibility of six *E. americana* strains to a variety of antimicrobial agents. Based upon MIC<sub>50</sub> data, at least some strains were found resistant to or had reduced susceptibility for ampicillin,  $\beta$ -lactamase-inhibitor combinations, cephalothin, cefoxitin, ticarcillin, chloramphenicol, doxycycline and trimethoprim; all six strains were susceptible to the aminoglycosides and to cefotaxime. *Ewingella americana* strains appear to have wide-

spread or universal resistance to ampicillin, first-generation cephalosporins and cephamycins, based upon individual case reports.

*Ewingella americana* appears to be a rare human pathogen, more frequently being associated with cases of polymicrobial bacteremia, pseudobacteremia, or nosocomial outbreaks of septicemia linked to contaminated catheters, ice baths, or coagulation tubes (Pien et al., 1983; Pien and Bruce, 1986; McNeil et al., 1987b). In most of these instances, the original source of *E. americana* appears to be water. The most common underlying theme of persons developing *E. americana* bacteremia has been surgery (Pien et al., 1983; Pien and Bruce, 1986; Devreese et al., 1992). In addition to bacteremia, *E. americana* has been associated with peritonitis in a woman with end-stage renal disease caused by polycystic kidney disease (Kati et al., 1999) and two ocular infections in immunocompetent adults (Heizmann and Michel, 1991; Da Costa et al., 2000). *Ewingella americana* also appears to be a possible causative agent of internal stipe necrosis (Inglis and Peberdy, 1996). This disease causes a browning disorder of the cultivated mushroom, *Agaricus bisporus*.

The type strain of *E. americana* is CDC 1468078 (= ATCC 33852 = CIP 8194).

## *Kluyvera*

Asai and colleagues (Asai et al., 1955) studied a group of strains from soil and sewage in Japan that produced large amounts of  $\alpha$ -ketoglutaric acid during fermentation of glucose. Six of their strains were peritrichously flagellated and assigned to *Escherichia* and "*Aerobacter*" (Brenner, 1991). Five other strains were thought to be polar flagellates and were placed in the new genus *Kluyvera* in the family Pseudomonadaceae. Two species, "*Kluyvera citrophila*" and "*Kluyvera noncitrophila*" were created based upon the ability to use citric acid (Brenner,

1991). Asai et al. (1962) subsequently confirmed the observation of Shewan and Hugh (cited in Asai et al., 1962) that *Kluyvera* species have peritrichous flagella (Brenner, 1991). They transferred them to *Escherichia*, thus effectively abolishing the genus *Kluyvera*. *Kluyvera* formally lost standing in nomenclature when it was omitted from the *Approved Lists of Bacterial Names* (Skerman et al., 1980).

From 1965 to 1980, some 145 strains that appeared biochemically similar to *Kluyvera* were received for identification at the CDC (Brenner, 1991). They were assigned to Enteric Group 8 (Farmer et al., 1981). DNA relatedness studies on 44 Enteric Group 8 strains indicated three closely related species (60–70% interspecies relatedness). Farmer et al. (1981) revived the genus name *Kluyvera* for these species. Both strains originally designated “*K. noncitrophila*” and two of three designated “*K. citrophila*” by Asai and colleagues were in the same DNA hybridization group (named “*K. cryocrescens*”; the other “*K. citrophila*” strain was in the hybridization group named “*K. ascorbata*”). Since the ability to utilize citrate did not correlate with the genomospecies, Asai’s species names were not revived (Brenner, 1991). One species was named “*Kluyvera ascorbata*” because it produced acid from ascorbate. A second species was named “*Kluyvera cryocrescens*” because it grew and fermented glucose at 5°C (Brenner, 1991). A third genomospecies (*Kluyvera* species 3), which contained five strains, was not named at that time. In 1996, a taxonomic study of *Buttiauxella* and *Kluyvera* strains primarily isolated from human sources and mollusks identified two new *Kluyvera* species (Müller et al., 1996). Strains formerly assigned to *Kluyvera* species 3 (Enteric Group 35/36) were designated “*Kluyvera georgiana*,” while a second previously unrecognized taxa was named “*Kluyvera cochleae*.” All strains within each newly designated species exhibited >70% interspecies relatedness at both 60 and 75°C (Müller et al., 1996). Relatedness to other members of the Enterobacteriaceae ranged from 15 to 30%. The G+C content of *Kluyvera* is 55–57 mol% (Farmer et al., 1981). Both *K. ascorbata* and *K. cryocrescens* produce the ECA (Böttger et al., 1987).

The major habitat(s) of *Kluyvera* species is presently unknown. The CDC culture collection contained 163 *Kluyvera* strains in 1985 (Farmer et al., 1985; Brenner, 1991), a majority of which were *K. ascorbata* (n = 123). Regardless of species designation, most *Kluyvera* strains of human origin were isolated from the respiratory tract, urine and stool in decreasing order of frequency. *Kluyvera* species have also been found in sewage, water, soil samples, a hospital sink, milk and a cow (Farmer et al., 1981). Recently, *Kluyvera*

species have been isolated from new environmental and animal sources. *Kluyvera* species have been isolated from birds including captive raptors (Bangert et al., 1988). Although not probably a major reservoir, *K. cochleae* strains have been recovered from mollusks including snails and a slug (Müller et al., 1996). A 1999 bacteriologic survey looked at the isolation of Enterobacteriaceae from fresh and frozen vegetables. Seven *Kluyvera* isolates were recovered from fresh Finnish and imported vegetables (Österblad et al., 1999).

*Kluyvera* species are typical members of the family Enterobacteriaceae. Good growth occurs at 30–36°C, with moderate to good growth occurring at 42°C (Müller et al., 1996). Most species do not require amino acids for growth, although some isolates require one or more vitamins (Farmer et al., 1981). Of the four species, *K. cochleae* is the least active metabolically (Müller et al., 1996). Biochemically, *Kluyvera* species most closely resemble *Buttiauxella* from which they must be separated. Both genera possess similar fatty acid patterns with 16:0, 17:0 *cyclo*, and 14:0 predominating (Kämpfer et al., 1997). While no single test clearly separates these two genera, they can be distinguished based upon several biochemical reactions listed in Table 11.

Identification of *Kluyvera* isolates to species level is a difficult process since a number of the most discriminatory tests are not routinely found in diagnostic laboratories. Brenner (1991) recommends several tests that are useful in separating the two most common species, *K. ascorbata* and *K. cryocrescens*, from one another (Table 12).

*Kluyvera cochleae* can be easily distinguished from any *Buttiauxella* or other *Kluyvera* species since it is indole-negative and Voges-Proskauer-positive (Müller et al., 1996). However, some strains (~33%) of *K. cochleae* are Voges-Proskauer-negative (Farmer, 1999). The most useful tests to separate *K. georgiana* from *K. ascorbata* and *K. cryocrescens* are for L-arginine

Table 11. Tests useful in separating *Kluyvera* and *Buttiauxella*.

Test	<i>Buttiauxella</i>	<i>Kluyvera</i>
Indole	– <sup>a</sup>	+ <sup>d</sup>
Acid from		
α-Methyl-D-glucopyranoside	– <sup>b</sup>	+
Sucrose	–	V
Raffinose	– <sup>c</sup>	+

Symbols: see footnote in Table 4.

<sup>a</sup>Except *B. noackiae* (V).

<sup>b</sup>Except *B. ferrugutiae* (V) and *B. noackiae* (V).

<sup>c</sup>Except *B. agrestis* (+), *B. brennerae* (+), and *B. izardii* (V).

<sup>d</sup>Except *K. cochleae* (–).

Data from Farmer (1999).

Table 12. Differential biochemical reactions of *K. ascorbata* and *K. cryocrescens*.

Test	<i>K. ascorbata</i>	<i>K. cryocrescens</i>
Ascorbate	+	-
D-Glucose fermentation, 5°C, 21 days	-	+
Irgasan susceptibility	R	S
Dulcitol fermentation	V	-

Symbols: see footnote in Table 4; S, sensitive; and R, resistant.

Data from Brenner (1991).

and 3-hydroxybenzoate utilization (Müller et al., 1996). Unfortunately, biochemical results on differential tests used to separate *K. ascorbata* from *K. cryocrescens* have not been reported for *K. georgiana* and *K. cochleae* with the exception of dulcitol. Some strains of *K. ascorbata* produce blue and red crystals, termed “kluuveramycin,” upon prolonged incubation at room temperature on trypticase soy agar (Farmer et al., 1981). *Kluyvera ascorbata* CDC strain 2221-78 (ATCC 33434) is a strong producer of kluuveramycin. One of two strains of *K. cryocrescens* has been found to produce pyrrolidonyl peptidase, while neither strain of *K. ascorbata* tested produced this activity (Chagla et al., 1993). Five strains of *K. ascorbata* have been tested for susceptibility to select antibiotics (Frenay et al., 1988). All five strains were found susceptible to ampicillin, ticarcillin, cefoxitin, cefotaxime, doxycycline, and to aminoglycosides; one of five strains was resistant to cephalothin and trimethoprim.

Because of the technical difficulty in identifying *Kluyvera* isolates to species, the exact role each species plays in human disease is largely unknown. West and others (West et al., 1998) have summarized 18 possible cases of *Kluyvera* infection published in the literature since 1980. Of these 18 cases, only 8 of the strains were identified to species level, with an equal distribution between *K. ascorbata* and *K. cryocrescens*. Six of the 18 cases involve diarrheal disease where the association of *Kluyvera* with gastroenteritis is tenuous at best. The issue is further clouded by the fact that *Kluyvera* has been recovered from the gastrointestinal tract of asymptomatic individuals (Fainstein et al., 1981). Most cases of extraintestinal *Kluyvera* infection have involved persons with underlying disease. Only three fatal infections have been reported, two involving the blood and one a case of peritonitis, and it is not clear how much the infection and underlying illness each contributed to these negative outcomes. Tristram and Forbes (1988) reported a case of urinary tract infection and sepsis in an 11-month-old infant with fever, vomiting and lethargy. The infant suffered from bilateral vesicoureteral reflux, and *Kluyvera* (species not

Table 13. Type strains of *Kluyvera* species.

Species	Type strain	Other designations
<i>K. ascorbata</i>	CDC 0648-74	ATCC 33433
<i>K. cryocrescens</i>	CDC 2065-78	ATCC 33435
<i>K. cochleae</i>	S 3/1-49	ATCC 51609; DSM 9406; serial no. 185
<i>K. georgiana</i>	CDC 2891-76	ATCC 51603; DSM 9409; serial no. 189

identified) was recovered from a suprapubic aspirate and from blood. Luttrell et al. described a soft tissue infection of the right forearm in a healthy 37-year-old woman. She apparently acquired her *Kluyvera* infection (species not identified) after cutting herself on the open lid of a can in the garbage. A case of *Kluyvera* pyelonephritis (species not identified) has also been described in a five-year-old girl where >100,000 cfu/ml were recovered from a urine sample (Dollberg et al., 1990); she had no underlying abnormalities. A case of fatal *Kluyvera* mediastinitis (species not identified) has been reported in a 74-year-old man with adult onset diabetes, hypertension, and end-stage renal disease (Sierra-Madero et al., 1990). After undergoing multiple medical procedures including a five-vessel saphenous vein coronary artery bypass and aortic valve replacement, he developed mediastinitis due to *Kluyvera*. *Kluyvera* was recovered from a sternal wound, the sputum, and the blood of this patient. Biochemical test results on this strain did not conform to known characteristics of either *K. ascorbata* or *K. cryocrescens*. Another fatal *Kluyvera* infection involved a case of *K. ascorbata* peritonitis in a 13-year-old girl with Friedreich’s ataxia and a one-year history of weight loss (Yogev and Kozlowski, 1990; West et al., 1998). *Kluyvera ascorbata* was recovered from multiple pre-mortem and post-mortem samples including endotracheal tube aspirates, peritoneal fluid, urine (>100,000 cfu/ml), subdiaphragmatic abscesses, and from lung tissue. The latest reported *Kluyvera* infection involved a soft tissue infection of the right middle finger of a 31-year-old man with insulin-dependent diabetes mellitus (West et al., 1998). He abraded his finger while using a chemical stripper on flooring with his bare hands. A painful blister resulted and *K. cryocrescens* was isolated on two separate occasions from debrided tissue.

The type strains of *Kluyvera* species are listed in Table 13.

## *Leclercia*

Tamura et al. (1986) proposed transferring *Escherichia adecarboxylata* to the newly created

*Leclercia*. *Leclercia adecarboxylata* gives + + – – reactions for the IMViC (indole, methyl red, Voges-Proskauer, and citrate) tests and negative reactions for lysine and ornithine decarboxylases and arginine dihydrolase (Brenner, 1991). It was first isolated from foods and named “*E. adecarboxylata*” by Leclerc in 1962, largely on the basis of its IMViC pattern, which is identical to that of *E. coli* (Brenner, 1991).

Little attention was paid to *Escherichia adecarboxylata* until several research groups attempted to clarify the *Enterobacter agglomerans*-*Erwinia herbicola* complex. Gavini et al. (1983b) found that *Escherichia adecarboxylata* strains formed a distinct phenotypic cluster (E<sub>5</sub>), separate from all other groups in the *Enterobacter agglomerans* complex. Izard et al. (1985) classified Gavini’s groups E<sub>5</sub>, E<sub>3</sub> and E<sub>2</sub>, and group XI of Brenner et al. (1984) as *Escherichia adecarboxylata* on the basis of DNA hybridization and similar electrophoretic patterns of soluble proteins. In their study, *Escherichia adecarboxylata* was over 60% related to *Enterobacter cloacae*, and they did not recommend taxonomic changes. Tamura et al. (1986) also found that *Escherichia adecarboxylata* was phenotypically distinct from *Enterobacter agglomerans* and that it was a single species by DNA hybridization. In their study, *Escherichia adecarboxylata* was 32% or less related to other Enterobacteriaceae and only 3–5% related to *Enterobacter cloacae*. On the basis of these findings, they transferred *Escherichia adecarboxylata* to *Leclercia* (Brenner, 1991). The marked difference in relatedness of *Leclercia adecarboxylata* to *Enterobacter cloacae* and to other Enterobacteriaceae obtained in the studies of Izard et al. (1985) and Tamura et al. (1986) must be resolved to assess the validity of the genus *Leclercia* (Brenner, 1991). The G+C content is 51–55 mol% (Izard et al., 1985; Tamura et al., 1986). Production of ECA has not been reported.

The first isolates of *Leclercia adecarboxylata* were from foods in France (Brenner, 1991). The 23 strains studied by Izard et al. (1985) were from drinking water (20 strains), humans (2 strains), and a plant source. Of 86 strains reported by Tamura et al. (1986), 58 were from humans including from human sputum, blood, urine, stool and wounds in decreasing order of frequency. *Leclercia* has also been isolated from imported vegetables (Österblad et al., 1999). It appears identical to Enteric Group 41 (Farmer et al., 1985; Temesgen et al., 1997).

*Leclercia adecarboxylata* is lysine- and ornithine-decarboxylase-negative, fails to produce arginine dihydrolase, and is H<sub>2</sub>S-negative. As such, it must be distinguished from other biochemically similar genera within the Enterobacteriaceae such as *Ewingella*, *Moellerella* and

*Rahnella* (Table 10). The type strain produces a yellow pigment, but most other strains do not (Brenner, 1991). *Leclercia adecarboxylata* colonies resemble those of *E. coli* on MacConkey, desoxycholate-lactose, and eosin-methylene blue agars, and the IMViC reactions are those of *E. coli* (Tamura et al., 1986; Brenner, 1991). Thus, it may be prudent to review any strains identified as decarboxylase-negative *E. coli* (Brenner, 1991). *Leclercia adecarboxylata* produces pyrrolidonyl peptidase (Chagla et al., 1993). From the limited number of reports available in the literature, *L. adecarboxylata* appears to be uniformly susceptible to most commonly administered antibiotics including the aminoglycosides, carbapenems, ampicillin, trimethoprim-sulfamethoxazole, fluoroquinolones and to the cephalosporins (Temesgen et al., 1997). Resistance to fosfomycin was detected in one isolate (de la Obra et al., 1999).

Temesgen and colleagues (Temesgen et al., 1997) have summarized the clinical significance of *L. adecarboxylata*. This involves only a handful of cases. *Leclercia adecarboxylata* was recovered from five patients at the Mayo Clinic between 1984 and 1995. For three of these patients, *L. adecarboxylata* was recovered as part of mixed microbial flora of wound infections involving an abrasion to the right calf while camping, a crush injury to the right foot, and a penetrating nail injury to a toe. The clinical significance of *L. adecarboxylata* in each of these instances could not be determined. A fourth case from this study involved the isolation of *L. adecarboxylata*, along with other Gram-negative flora, from the sputum of a patient with right lower lobe pneumonia. The significance was highly questionable because of the large number of other microbial pathogens isolated simultaneously; however, Temesgen et al. (1997) did describe a case of *L. adecarboxylata* bacteremia in a 35-year-old woman with acute nonlymphocytic leukemia who had recently undergone a bone marrow transplantation. Two other bacteremia cases cited in this review, that of a 45-year-old man with ethanol cirrhosis and an 8<sup>1</sup>/<sub>2</sub>-month-old baby with congenital gastroschisis and intestinal atresia, support the rare role of *L. adecarboxylata* as a bloodborne pathogen. de la Obra et al. (1999) documented a fourth case of *Leclercia* bacteremia in a 42-year-old woman with multiple myeloma who presented with fever after apheresis. *Leclercia adecarboxylata* has also been associated with a case of peritonitis in a 5-year-old boy with end-stage renal disease (Fattal and Deville, 2000). The most recent episodes of *L. adecarboxylata* infection involve a chronically inflamed gallbladder in an elderly woman, and polymicrobial sepsis in an 11-month-old child with acute lymphoblastic leukemia and in an 80-

year-old woman with cardiovascular disease (De Baere et al., 2001; Longhurst and West, 2001). These collective reports indicate that *L. adedecarboxylata* is a very rare human pathogen primarily infecting persons that are immunocompromised or with severe underlying illnesses.

The type strain of *L. adedecarboxylata* is ATCC 23216.

## Leminorella

The genus *Leminorella* was created from 11 H<sub>2</sub>S-positive strains previously referred to as “Enteric Group 57” (Hickman-Brenner et al., 1985b; Brenner, 1991). DNA relatedness studies showed three genomospecies. *Leminorella grimontii* contained six strains, three from human stools, one from a mouse stool, and two from urines; *Leminorella richardii* contained four human strains, all from stools; and *Leminorella* species 3 contained a single strain from a human stool. The two named species were 32–49% inter-related (Brenner, 1991). *Leminorella* species 3 was 60% related to *L. grimontii* and 40% related to *L. richardii* (Brenner, 1991). Phylogenetic analysis of the type strain of *L. grimontii* indicates that *Leminorella* forms a distinct lineage in the family, well separated from other Enterobacteriaceae genera (Spröer et al., 1999). The cellular polyamine composition of both species includes acetylspermidine, putrescine, diaminopropane and spermidine (Hamana, 1996). The G+C content of *L. grimontii* is 52 mol%, that of *L. richardii* is 53 mol%, and that of species 3 is 55 mol% (Brenner, 1991). Both *L. grimontii* and *L. richardii* express the ECA (Böttger et al., 1987).

*Leminorella* is H<sub>2</sub>S-positive and must be separated from other rarely encountered H<sub>2</sub>S-positive genera in the family Enterobacteriaceae (Table 4). Members of the genus are lysine- and ornithine-decarboxylase negative and arginine dihydrolase negative. *Leminorella* resembles *Proteus* in its positive H<sub>2</sub>S reaction and tyrosinase activity and failure to ferment D-mannose, but it is easily distinguished from *Proteus* by its negative urea and phenylalanine deaminase reactions. Biochemically, *Leminorella* most closely resembles *Budvicia* and *Pragia*, however, *Pragia* is motile at 36°C, while *Leminorella* is not (Farmer, 1999). The ability of *Budvicia* to ferment L-rhamnose separates this genus from *Leminorella*. Some strains react weakly in *Salmonella* antisera (Brenner, 1991). *Leminorella* species can be separated from one another on the basis of the following tests in Table 14.

Until recently the clinical significance of *Leminorella* was in doubt (Brenner, 1991). However a 28-month study at the Tel Aviv Sourasky Med-

Table 14. Differentiation of *L. grimontii* and *L. richardii*.

Test <sup>a</sup>	<i>L. grimontii</i>	<i>L. richardii</i>
Citrate (Simmons')	+	–
Methyl red	+	–
Acid from dulcitol	V	–

Symbols: see footnote in Table 4.

<sup>a</sup>Reactions are for 7 days.

Adapted from Hickman-Brenner et al. (1985) and Farmer (1999).

Table 15. Type strains of *Leminorella* species.

Species	Type strain	Other designations
<i>L. grimontii</i>	CDC 1944-81	ATCC 33999
<i>L. richardii</i>	CDC 0978-82	ATCC 33998

ical Center revealed 18 patients from whom *Leminorella* was isolated for an overall incidence of 11 cases/100,000 admissions (Blekher et al., 2000). For 14 of these patients, medical records were available for review. Using modified CDC criteria for determining clinical significance, five patients were judged to have definite infections caused by *Leminorella* species. Two of these infections were associated with the urinary tract (>100,000 cfu/ml, pyuria), one wound infection involved a fractured tibia, a case of peritonitis in a 75-year-old man, and a case of primary bacteremia in a 10-year-old boy with acute myelocytic leukemia (Blekher et al., 2000). The average age of these 14 patients was 67 years, and most illnesses involved males (78%) and were nosocomial in origin (93%). All patients had other comorbid conditions. In addition to these definite cases (43%), another 50% of the patients were thought to have probable or possible infections caused by *Leminorella* (Blekher et al., 2000).

The type strains of *Leminorella* are listed in Table 15.

## Moellerella

In 1984, a group of nine strains previously assigned to Enteric Group 46 by the CDC were shown to exhibit 80–93% interspecies DNA relatedness at 60°C and 78–97% relatedness at 75°C using the hydroxyapatite method (Hickman-Brenner et al., 1984). No other group within the family Enterobacteriaceae, including 49 named and unnamed species, was more than 32% related (*Proteus* and *Providencia*) at 60°C to Enteric Group 46. Even *Proteus* and *Providencia* were only 4–7% related to Enteric Group 46 at the more stringent temperature of 75°C.

Based upon these findings, Enteric Group 46 represented a new genus in the family Enterobacteriaceae and the name *Moellerella wisconsensis* was proposed (Hickman-Brenner et al., 1984). *Moellerella wisconsensis* expresses ECA (Böttger et al., 1987). The G+C content of *M. wisconsensis* has not been determined (Brenner, 1991).

The natural habitat of *M. wisconsensis* is unknown. Most strains have been recovered from human feces. In the original description of the genus, 8 of 9 isolates were of fecal origin, with the remaining strain recovered from drinking water (Hickman-Brenner et al., 1984). Marshall et al. (1986) screened 400 stool samples for *M. wisconsensis* and isolated three additional strains (0.75%).

Medium of Marshall et al. (1986) for the Recovery of *M. wisconsensis*

Proteose peptone No. 3 (Difco)	20.0 g
Lactose	10.0 g
Neutral red	0.075 g
Sodium chloride	5.0 g
Agar	12.0 g
Polymyxin	B30.0 g
Bacitracin	500,000 IU

Dilute up to 1 liter with deionized water and adjust pH.

Other human sources from which *M. wisconsensis* has been isolated include a wound, gall bladder, and respiratory secretions (Wittke et al., 1985; Wallet et al., 1994). *Moellerella wisconsensis* has also been isolated from four captive raptors fed chicken-containing diets (Bangert et al., 1988).

*Moellerella wisconsensis* is indistinguishable from *E. coli* on MacConkey and eosin-methylene blue agars producing deep pink and dark colonies with a green metallic sheen, respectively (Hickman-Brenner et al., 1984). *Moellerella wisconsensis* has a - + - + IMViC pattern and is negative in tests for urea, phenylalanine-, lysine- and ornithine decarboxylases, arginine dihydrolase, motility, and gas from D-glucose (Hickman-Brenner et al., 1984; Brenner, 1991). Most strains are susceptible to cephalothin, chloramphenicol and gentamicin, and resistant to ampicillin, carbenicillin and colistin; however, definitive susceptibility studies on this bacterium are lacking. *Moellerella* needs to be separated from other H<sub>2</sub>S-negative bacteria with similar biochemical characteristics including *Ewingella*, *Tatumella* and *Photorhabdus/Xenorhabdus* (Table 10).

The pathogenicity of *M. wisconsensis* is unknown. Hickman-Brenner et al. (1984) described one case of bloody diarrhea in a 40-year-old physician who developed fever, abdominal pain, and cramps within 48 hours of his arrival in Peru. His stool culture yielded predom-

inant growth of *M. wisconsensis*. Another possible case of infection involved a 71-year-old man with acute cholecystitis that resulted in the isolation of *M. wisconsensis* along with enterococci in approximately equal numbers from a swab of his gallbladder (Wittke et al., 1985). A definite pathogenic role for *M. wisconsensis* could not be established. Although unproven, it is likely that *M. wisconsensis* can cause disease in susceptible hosts in a fashion similar to that recorded for other rarely encountered genera of Enterobacteriaceae listed in this chapter.

The type strain of *M. wisconsensis* is CDC 2896-78 (= ATCC 35017).

## *Obesumbacterium*

The genus has a checkered taxonomic history dating back to 1936 when it was first isolated from top-fermentation pitching-yeast samples associated with the brewing process (Shimwell, 1964). The genus is almost exclusively associated with breweries and the brewing process and most studies concerning *Obesumbacterium proteus* have been reported in brewery journals (Brenner, 1991). For a history of nomenclature and taxonomic changes associated with the genus *O. proteus*, please consult the extensive review of Farmer (1984).

In 1964, Shimwell proposed creation of a new genus, *Obesumbacterium*, to house short fat rods that were commonly found in breweries and had previously been designated "*Flavobacterium proteus*." This proposal was based upon the facts that these organisms did not show any relationship to the genus "*Flavobacterium*" and did not fit into any other currently recognized genera. Thus, creation of a new genus was warranted and the name "*Obesumbacterium*" was proposed. Priest et al. (1973) demonstrated that *O. proteus* strains formed two phenotypic groups, both of which were compatible with inclusion in the family Enterobacteriaceae (Brenner, 1991). DNA hybridization studies, while not very discriminatory, indicated that that *O. proteus* was significantly related to the Enterobacteriaceae, and in particular to *Hafnia alvei* (Brenner, 1991). On the basis of these findings, Priest et al. (1973) proposed transferring *O. proteus* to *Hafnia*, as "*Hafnia protea*." *Hafnia protea* was accepted and used by microbiologists in the brewery industry (Farmer, 1984). However, this name lost standing in nomenclature when it failed to appear on the *Approved Lists of Bacterial Names* (Skerman et al., 1980). *Obesumbacterium proteus* was on the *Approved List* and remains the accepted name (Brenner, 1991).

Brenner (1981) confirmed that *O. proteus* belonged in the Enterobacteriaceae and showed

Table 16. DNA relatedness of *O. proteus* biogroups.

Unlabeled DNA	Source of labeled DNA							
	<i>O. proteus</i> biogroup 2		<i>O. proteus</i> biogroup 1		<i>H. alvei</i> genomospecies 1		<i>H. alvei</i> genomospecies 2	
	60°C <sup>a</sup>	D	60°C <sup>a</sup>	D	60°C <sup>a</sup>	D	60°C <sup>a</sup>	D
<i>O. proteus</i> biogroup 2	92–96	0	24–25	12–14.5	22–26	NT	16–20	NT
<i>O. proteus</i> biogroup 1	26–33	NT	92–100	0–2	73–77	3.5–66	45–56	NT
<i>H. alvei</i> genomospecies 1	28	18	71–73	3.5–4	81–100	0–1	58–62	12
<i>H. alvei</i> genomospecies 2	27–28	18	54–55	11.5	58	12–13	88–100	0–0.5

Abbreviations: D, divergence within related DNA sequences, expressed to nearest 0.5%; and NT, not tested.

<sup>a</sup>Percent relatedness at 60°C.

Adapted from Brenner (1991).

Table 17. Differential characteristics of *Obesumbacterium proteus* and *Hafnia alvei*.

Test <sup>a</sup>	<i>O. proteus</i>		<i>H. alvei</i> <sup>b</sup>	
	Biogroup 1	Biogroup 2	Genomospecies 1	Genomospecies 2
<i>Hafnia</i> phage lysis	+	–	+	+
Voges-Proskauer	+	–	+	+
Gas from D-glucose	–	–	+	+
Growth in KCN	V	–	+	+
Acid from				
Salicin	+	–	V	–
D-Mannitol	+	–	+	+
D-Xylose	–	+	+	+

Symbols: see footnote in Table 4.

<sup>a</sup>Reactions are for 7 days.

<sup>b</sup>*H. alvei* DNA hybridization groups cannot be unambiguously separated from one another biochemically.

Adapted from Brenner (1981, 1991) and Farmer (1999).

that the two biogroups were in fact separate species (Table 16).

*Obesumbacterium proteus* biogroup 1 is an anaerogenic biogroup of *H. alvei* DNA hybridization group 1 (Brenner, 1991). Biogroup 2 was related to *Escherichia blattae* (50–60% related at 60°C) and represents a new genomospecies within *Escherichia*. Unfortunately, the type strain of *O. proteus* (Priest 502 = CDC 4296-74 = NCIB 8771 = ACTT 12841) resides in *Obesumbacterium* biogroup 1 (Brenner, 1991), and therefore, by definition, is *H. alvei*, since *H. alvei* genomospecies 1 contains the type strain ATCC 13337 (= NCTC 8105 = CDC 434-68) of *H. alvei*. The type strain of *O. proteus* shares 99.5% 16S rDNA sequence similarity with *H. alvei* ATCC 13337 (Spröer et al., 1999). This means that there is no valid type strain for *O. proteus*. The high degree of DNA relatedness between *O. proteus* biogroup 2 and *E. blattae* brings into question the legitimacy of the genus *Obesumbacterium* and whether biogroup 2 should be transferred to the genus *Escherichia* (Brenner, 1991). The G+C content of *O. proteus* is 49 mol% (Priest et al., 1973). Both *O. proteus* biogroups produce ECA (Ramia et al., 1982).

*Obesumbacterium proteus* is a pleomorphic, nonmotile bacterium (0.8–2.0  $\mu$ m in width and 1.5–100  $\mu$ m in length), ranging in shape from short plump rods to long pleomorphic bacilli (Farmer, 1984). *Obesumbacterium* grows slowly with colonies <0.5 mm in size after overnight incubation on most common media (Farmer, 1984). *Obesumbacterium proteus* produces acid from the fermentation of D-glucose and D-mannose and is lysine decarboxylase-positive. Biogroup 1 differs from the two DNA relatedness groups of *H. alvei* in that it fails to produce gas from the fermentation of D-glucose and does not ferment D-xylose (Brenner, 1991). As with both DNA hybridization groups of *H. alvei*, biogroup 1 of *O. proteus* is susceptible to the *Hafnia*-specific bacteriophage 1672 of Guinée and Valkenburg (1968). *Obesumbacterium proteus* biogroup 2 is easily separable from *H. alvei* and from *O. proteus* biogroup 1 by its lack of susceptibility to the *Hafnia*-specific bacteriophage, its inability to ferment D-mannitol, and its negative Voges-Proskauer reaction (Brenner, 1991). Salient biochemical features are listed in Table 17.

*Obesumbacterium* is exclusively isolated from breweries, beer wort, and the brewing fermenta-

tion process. There are no clinical isolates of *O. proteus*.

There is no valid type strain of *O. proteus* at present.

## *Pantoea*

The genus *Pantoea* was created in 1989 by Gavini and colleagues to house two closely related taxa that had formerly resided in several genera, including *Enterobacter* and *Erwinia*. One taxon, referred to as “DNA hybridization group (HG) 27155” by Beji and others (Beji et al., 1988) consisted of the type strain of *Enterobacter agglomerans* (ATCC 27155) and strains belonging to the B4 phenom of Gavini et al. (1983a). An electrophoretic protein analysis of these strains indicated that those belonging to protein profile group 1, which correlated with phenoms 7B and 8 of Verdonck et al. (1987), were highly related at the DNA level with a minimum DNA relatedness of 81% (Beji et al., 1988). Several other protein groups (III–VI) were also demonstrated by DNA-DNA hybridization to be 82–96% related to ATCC 27155. Also, HG 27155 appeared identical to Brenner’s HG XIII since three HG XIII strains of Brenner were 94–95% related to ATCC 27155 (Brenner et al., 1984). Included within HG 27155 were the type strains of *E. agglomerans* (ATCC 27155), *Erwinia herbicola* (NCPPB 2971) and *Erwinia milletiae* (NCPPB 2519). The second taxon, referred to as “DNA HG 14589” consisted of five strains from Gavini’s B5 phenom, three strains from Verdonck’s phenom 10, CDC 1429-71 from Brenner’s HG III, and *Erwinia herbicola* ATCC 14589. The closest relative of HG 14589 was HG 27155, which exhibited 41–53% DNA relatedness as determined by DNA-DNA hybridization (Gavini et al., 1989). HG 14589 was only 28% related to the type strains of *Erwinia caratovora* and *Erwinia amylovora* and 1–37% related to 37 other genera and species in the family Enterobacteriaceae (Gavini et al., 1989). These data indicated that both HG 27155 and HG 14589 were discrete species in the family Enterobacteriaceae and should be placed in a separate genus. Gavini et al. (1989) proposed the genus *Pantoea* for these two taxa. The species name “*agglomerans*” has priority in the literature over the epithets *herbicola* and *milletiae* according to the *International Code of Nomenclature of Bacteria* edited by Sneath (1992). *Enterobacter agglomerans* (HG 27155) was therefore transferred to the new genus as *Pantoea agglomerans* (Gavini et al., 1989). HG 14589 was named *P. dispersa*.

A Japanese study of bacterial strains that produced 2,5-diketo-D-gluconic acid from D-glucose placed 42 of these isolates into the genus *Erwinia*

as “*Erwinia citreus*,” “*Erwinia punctata*” and “*Erwinia terreus*” (Kageyama et al., 1992). Subsequent numerical taxonomy studies performed on 37 of these isolates identified three phenoms represented by strains SHS 2003, SHS 2006 and SHS 2008. Using simple matching coefficients, these three phenoms displayed the greatest similarity (68.4%) to *P. agglomerans* and *P. dispersa*. Group SHS 2003 was 28–41% related to groups SHS 2006 and SHS 2008 by DNA-DNA hybridization (S1 nuclease, 60°C). Group SHS 2006 was 38–46% related to group SHS 2008. Interspecies relatedness of four SHS 2006 strains and four SHS 2008 strains was 85–100% and 71–99%, respectively (Kageyama et al., 1992). The DNA relatedness of each of these groups to other strains of the *Erwinia herbicola-Enterobacter agglomerans* complex, other *Erwinia* and *Enterobacter* species, and selected genera in the family Enterobacteriaceae was <20%. Relatedness to *P. agglomerans* and *P. dispersa* was 9–18%. Based primarily on phenotypic relatedness and numerical taxonomy studies, which produced a closest match with the genus *Pantoea*, Kageyama et al. (1992) proposed the names “*P. citrea*,” “*P. punctata*” and “*P. terrea*” for groups represented by strains SHS 2003, SHS 2006 and SHS 2008, respectively. Two additional DNA HGs were subsequently detected by Mergaert et al. (1993). HG 2665 contained the type strains for *Erwinia ananas* and *Erwinia ure-dovora* plus five other *Erwinia* strains representative of protein profile groups I to V. The interspecies relatedness of these 7 strains was 76–100% as determined by DNA-DNA hybridization (renaturation measured spectrophotometrically, 74°C). Mergaert et al. (1993) proposed that HG 2665 be transferred to the genus *Pantoea* as *P. ananas*, since the epithet *ananas* has priority in the literature over *ure-dovora*. A second HG, which consisted of six *Erwinia* isolates, including three *E. stewartii* strains, represented protein profile groups VI–IX. Two subgroups existed within this HG-labeled subgroup, 2632 and 2715. Subgroup 2715 contained the type strain of *Erwinia stewartii*. Intergroup relatedness was >93%. Subgroups 2632 and 2715 were 60–83% related to each other, but only 30–39% related to *E. ananas* (HG 2665). Based on these DNA relatedness values, the second HG was transferred to the genus *Pantoea* as *P. stewartii* with subgroup 2715 as *P. stewartii* subsp. *stewartii* and 2632 as *P. stewartii* subsp. *indologenes* (Mergaert et al., 1993).

In a phylogenetic study of 29 plant-associated strains based upon 16S rDNA sequences, four *Pantoea* species, including the type strains of *P. ananas* and *P. stewartii* formed a monophyletic unit (cluster IV), closely related to *Erwinia* (Hauben et al., 1998). The 16S rDNA sequence



similarities for these four strains ranged from 95.9 to 98.8% with an average similarity of 96.1% with *Erwinia* species. The G+C content of the genus *Pantoea* was originally reported as 55.1–60.6 mol% (Gavini et al., 1989); however, the G+C content for *P. punctata*, *P. citrea* and *P. terrea* is 50–50.3, 49.7 and 51.0–51.9 mol%, respectively (Kageyama et al., 1992), which has led to an amended description of the genus *Pantoea* (Mergaert et al., 1993). *Pantoea agglomerans*, *P. ananas* and *P. stewartii* express ECA (Böttger et al., 1987).

Members of the genus *Pantoea* are widely distributed in nature occurring in many different ecologic habitats, including in association with plants, soil, water, as well as with humans and other animals (Gavini et al., 1989). Plant surfaces, seeds, water, clinical samples, animals, and raw and frozen vegetables harbor *P. agglomerans* (Gavini et al., 1989; Iimura and Hosono, 1996; Österblad et al., 1999). *Pantoea dispersa* has been recovered from plant surfaces, seeds and humans (Gavini et al., 1989). *Pantoea citrea*, *P. punctata* and *P. terrea* have been isolated from Japanese fruit, particularly mandarin oranges (Kageyama et al., 1992); *P. terrea* has also been isolated from soil. *Pantoea ananas* has been isolated from pineapple, rice seeds, and from the wheat fungal pathogen *Puccinia graminis* (Mergaert et al., 1993). *Pantoea stewartii* has been isolated from grasses, pineapple and beetles.

Members of the genus *Pantoea* share most of the classic phenotypic characteristics associated with genera in the family Enterobacteriaceae. For most *Pantoea* species, good growth occurs over a temperature range of 20–36°C. Almost 90% of *P. ananas* strains grow at 4°C, but not most other *Pantoea* species with the exception of *P. stewartii* subsp. *indologenes* (Mergaert et al.,

1993). In contrast, *P. dispersa* grows at 41°C, while most other *Pantoea* species do not (Gavini et al., 1989). *Pantoea citrea*, *P. punctata* and *P. terrea* grow at a pH range of 6.0–7.5 and all three require nicotinamide or nicotinic acid for growth (Kageyama et al., 1992). Biochemically, all *Pantoea* species are negative for lysine and ornithine decarboxylase and arginine dihydrolase. Thus, they need to be separated from other H<sub>2</sub>S-negative genera in the Enterobacteriaceae (see Table 10). Although *Pantoea* species are H<sub>2</sub>S-negative on traditional media such as triple-sugar, iron-agar slants, *P. citrea*, *P. punctata* and *P. terrea* are H<sub>2</sub>S-positive in cysteine-based media (Kageyama et al., 1992). Salient phenotypic tests useful in determining the species designation of individual *Pantoea* isolates are provided in Table 18.

*Pantoea* species are primarily plant pathogens. *Pantoea agglomerans* has been associated with the gall formation on *Wisteria* species and on Baby's breath (*Gypsophila paniculata*). It has also been implicated in stalk and leaf necrosis of onions (Gavini et al., 1989). On the leaves of beach peas (*Lathyrus maritimus*), it causes black spot necrosis (Khetmalas et al., 1996). *Pantoea dispersa* has been associated with leaf spot in okra (*Abelmoschus esculentus*). *Pantoea citrea* appears to cause pink disease of pineapples (Pujol and Kado, 2000). The disease is characterized by a distinct orange-brown color produced in fruit tissue after the heating process of canning. Discoloration appears to be due to production of 2, 5-diketogluconate by *P. citrea*. *Pantoea stewartii* subsp. *stewartii* causes the vascular disease Stewart's bacterial wilt of corn (*Zea mays*). *Pantoea stewartii* subsp. *indologenes* causes leaf spot in foxtail and pearl millet (Mergaert et al., 1993). It also causes pineapple rot (*Ananas comosus*).

Table 18. Phenotypic characteristics of *Pantoea* species.

Test <sup>a</sup>	<i>P.</i>	<i>P.</i>	<i>P.</i>	<i>P.</i>	<i>P.</i>	<i>P.</i>	<i>P. stewartii</i> subsp.	
	<i>agglomerans</i>	<i>dispersa</i>	<i>citrea</i>	<i>punctata</i>	<i>terrea</i>	<i>ananas</i>	<i>stewartii</i>	<i>indologenes</i>
Yellow pigment	+	V	–	–	–	+	+	+
Motility	+	+	–	–	+	+	–	V
Growth, 41°C	–	+	–	–	–	V	–	V
Malonate	+	–	–	–	–	–	–	–
PDA	+	–	–	–	–	–	–	–
Indole	–	–	–	–	–	+	–	+
ONPG	+	+	+	–	–	+	+	+
Acid from								
Melibiose	–	V	+	+	+	+	+	+
Sorbitol	–	–	V	–	V	V	–	–
α-Methyl-D-mannoside	–	–	–	–	–	+	–	–

Symbols: see footnote in Table 4.

Abbreviations: PDA, phenylalanine deaminase; and ONPG, *o*-nitrophenyl-β-galactopyranoside.

<sup>a</sup>Reactions are at 30°C.

Adapted from Gavini et al. (1989), Kageyama et al. (1992) and Mergaert et al. (1993).

Table 19. Type strains of *Pantoea* species.

Species	Type strain	Other designations
<i>P. agglomerans</i>	ATCC 27155	CDC 1461-67; NCTC 9381; LMG 1286; ICPB 3435
<i>P. dispersa</i>	ATCC 14589	LMG 2603
<i>P. citrea</i>	SHS 2003	ATCC 31623
<i>P. punctata</i>	SHS 2006	ATCC 31626
<i>P. terrea</i>	SHS 2008	ATCC 31628
<i>P. ananas</i>	LMG 2665	NCPBP 1846; ATCC 33244; PDDCC 1850; ICPB EA175
<i>P. stewartii</i>	LMG 2715	NCPBP 2295; ATCC 8199; ICMP 257; DSM 30176; ICPB SS11; IMET 11187

Because of the confusing taxonomy and the difficulty in identifying *Pantoea* isolates to species, little is presently known about their relative pathogenicity in humans, although it is assumed to be low. *Pantoea agglomerans* has been isolated from leg wounds, a knee exudate, sputum, trachea, spinal fluid, urine, conjunctiva and peritoneal dialysis fluid (Janda and Abbott, 1998). The older literature contains numerous reports of the recovery of “*Enterobacter agglomerans*” linked to outbreaks of pseudobacteremia associated with contaminated medical devices or solutions (Astagneau et al., 1994). “*Enterobacter agglomerans*” has also been linked to “cotton fever,” an acute febrile condition associated with postinjection of intravenous heroin in narcotic abusers using cotton as a filter for heroin (Ferguson et al., 1993). De Champs et al. (2000) have recently described two cases of septic monoarthritis caused by *P. agglomerans* after penetrating injuries caused by a thorn and a wood sliver. The role other *Pantoea* species in human disease is presently unknown.

The type strains of *Pantoea* species are listed in Table 19.

## *Pectobacterium*

The genus *Pectobacterium* was revived by Hauben et al. in 1998 during a study of phytopathogenic strains belonging to the family Enterobacteriaceae (Hauben et al., 1998). In that investigation, the phylogenetic position of these isolates was assessed using almost complete 16S rDNA sequences for 29 plant-associated strains. Construction of a neighbor-joining dendrogram identified one cluster (Cluster II) consisting of five subspecies of *Erwinia caratovora*, *E. cacticida*, *E. chrysanthemii* and *E. cyripedii*. The mean 16S rDNA sequence similarity value for these eight strains was 96.8%. The sequence similarity of this cluster to the genera *Erwinia*, *Brenneria* and *Pantoea* ranged from 95.3 to 95.6%. Sequence divergence within the subspecies of *E. caratovora* varied from 1.2 to 3.0%. Strains of *E. caratovororum*, *E. chrysanthemii* and *E. cyripedii* had previously been shown to be only 16–22%

related to *E. amylovora* by DNA binding (Brenner et al., 1974). Cluster II exhibited only 95.6% similarity to true erwinias, while *Pantoea* (cluster IV) demonstrated only an average of 96.1% 16S rDNA sequence similarity with *Erwinia*. Based upon this latter fact, Hauben et al. (1998) proposed that cluster II should be considered a distinct genus and supported the revival of the genus *Pectobacterium*, which originally included pectinolytic Enterobacteriaceae (Waldee, 1945). *Pectobacterium* contains signature nucleotides G, C, T, T, G, C, A, A, C, G, C, C, T, A, G, T, T and A at sequence positions 408, 434, 594, 598, 599, 638, 639, 646, 839, 847, 848, 988, 989, 1216, 1217, 1218, 1308 and 1329 according to the *E. coli* 16S rRNA gene sequence (Hauben et al., 1998). Spröer et al. (1999) has subsequently conducted phylogenetic studies on 15 genera within the family Enterobacteriaceae, including three species of *Pectobacterium*. In contrast to both Kwon et al. (1997) and Hauben et al. (1998), these authors did not find that *Pectobacterium* formed a phylogenetically well separated cluster, but rather depicted the origin of *Brenneria* species within the radiation of *Pectobacterium* species (Spröer et al., 1999). The suggestion that the genus *Pectobacterium* is composed of a polyphyletic group is supported by nucleotide sequence studies on a 447-bp segment of the *gapDH* locus, which encodes for the glyceraldehyde-3-phosphate dehydrogenase (Brown et al., 2000). Studies of *gapDH* sequence evolution indicate that *P. chrysanthemii* and *P. cacticidum* have evolved separately from the *P. caratovororum* clade. The differences in conclusions need to be addressed. The G+C content for *Pectobacterium* species is 50.5–54.6 mol% (Hauben et al., 1998). *Pectobacterium caratovororum* and *P. cyripedii* produce the ECA, while *P. chrysanthemii* does not (Böttger et al., 1987).

*Pectobacterium* species were formerly classified in the “caratovora” group of the genus *Erwinia*. With the exception of *P. cyripedii*, *Pectobacterium* species produce strong pectinolytic activity and cause soft rot, necrosis, and wilts on crops and ornamental plants (Hauben et al., 1998). These species cause soft rot in a wide range of vegetables and plants including pota-

Table 20. Phenotypic characteristics of *Pectobacterium* species.

Test	<i>P. cacticidum</i>	<i>P. carotovorum</i>	<i>P. chrysanthemi</i>	<i>P. cyripedii</i>
Pectinase	+	+	+	–
Growth at 43°C	+	–	–	ND
Erythromycin sensitivity	–	–	+	+
Indole	–	+	V	–
Malonate	+	–	+	ND
Acid from D-Raffinose	–	+	+	–

Symbols: see footnote in Table 4; and ND, not determined.

Adapted from Alcorn et al. (1991) and Hauben et al. (1998).

Table 21. Type strains of *Pectobacterium* species.

Species	Type strain	Other designations
<i>P. cacticidum</i>	1–12	ATCC 49481; LMG 17936
<i>P. carotovorum</i>	NCPPB 312	ATCC 15713; BS 1008; ICMP 5702; LMG 2404
<i>P. chrysanthemi</i>	EC17	ATCC 11663; ICMP 5703; NCPPB 402; LMG 2804
<i>P. cyripedii</i>	ICPB EC 155	ATCC 29267; ICMP 1591; NCPPB 3004; LMG 2657

toes, carrots, onions, sugarbeets, sweet potatoes, papayas and cabbage (Sinha and Prasad, 1977; Hauben et al., 1998).

The genus *Pectobacterium* possesses the defining phenotypic features of the family Enterobacteriaceae. All species are negative for lysine-, ornithine decarboxylase and arginine dihydrolase, and thus, must be separated from phenotypically similar genera, including *Erwinia*, *Brenneria*, *Pantoea* and other unusual genera in the family Enterobacteriaceae (see Table 10). They fail to deaminate tryptophan and are urea-negative but hydrolyze esculin. Additional biochemical features of the genus *Pectobacterium* can be found in Hauben et al. (1998). Key differential traits useful in identifying *Pectobacterium* isolates to species level are listed in Table 20.

*Pectobacterium* species cause a variety of plant diseases. Various subspecies of *P. carotovorum* causes storage rot in potato tubers, vascular necroses in roots of sugarbeets, rotting diseases on Japanese horseradish, and slimy rot of witloof chicory (Hauben et al., 1998). *Pectobacterium chrysanthemi* causes vascular wilts or parenchymal necroses in corn and other food crops (Hauben et al., 1998). *Pectobacterium cacticidum* is found in soft rot tissues of cacti (Alcorn et al., 1991). *Pectobacterium cyripedii* causes brown rot in orchids (Hauben et al., 1998).

The type strains of *Pectobacterium* species are listed in Table 21.

## *Photorhabdus*

The genus *Photorhabdus* was proposed in 1993 to include bacterial endosymbionts of entomopathogenic nematodes that had previously

been assigned to the genus *Xenorhabdus* as *X. luminescens* (Boemare et al., 1993). Creation of this new genus appeared warranted since *X. luminescens* strains were significantly different from all other known *Xenorhabdus* strains at that time on a genetic, phenotypic, physiologic and ecologic basis (Boemare et al., 1993). DNA relatedness studies conducted by Grimont et al. (1984) demonstrated only low intraspecies DNA relatedness values between *X. nematophilus* and *X. luminescens* (8–12%, S1 nuclease method; 17–37%, hydroxyapatite method). Subsequent studies using the hydroxyapatite method confirmed these earlier observations. The DNA of *X. luminescens* strains was found to have relative binding ratios of only 5–23% to other nonluminescent members of *Xenorhabdus* (Boemare et al., 1993). RNase T1-resistant oligonucleotide 16S rRNA cataloguing of *X. luminescens* ATCC 29999 and ATCC 29304 yielded a similarity coefficient of 0.71 to *X. nematophilus* ATCC 19061, indicating only moderate relatedness between these two species (Ehlers and Stackebrandt, 1988). These two groups are further distinguished by the presence in *X. luminescens* of more branched chain fatty acids (C<sub>1-15</sub>, C<sub>1-17</sub>) and a smaller percentage (<10%) of cyclopropane acid (C<sub>17-cy</sub>; Janse and Smits, 1990; Suzuki et al., 1990). In addition to genetic investigations, *X. luminescens* is catalase-positive and bioluminescent, while all other *Xenorhabdus* strains are catalase-negative and nonluminescent. Furthermore, bioluminescent bacteria are known to be endosymbionts of the family Heterorhabditidae, while other nonluminescent members of the genus *Xenorhabdus* are primarily associated with nematodes of the family Steinernematidae (Akhurst et al., 1996). Based upon this collective

data, Boemare et al. (1993) transferred *X. luminescens* to a new genus, *Photorhabdus*. The genus name, which means “light rod” is reflective of the unique phenotypic attribute of this species.

Although the genus *Xenorhabdus* appears to be more diverse than the genus *Photorhabdus* (Fischer-Le Saux et al., 1998), early studies indicated that more than one DNA relatedness group existed within *P. luminescens* (Grimont et al., 1984). DNA relatedness studies (hydroxyapatite method) conducted on 31 *P. luminescens* strains found two distinct DNA groups, one composed of symbiont strains and one composed of clinical isolates (Akhurst et al., 1996). A polyphasic taxonomic survey employing DNA pairing (S1 method) studies, 16S rRNA inferences, and phenotypic characteristics of 23 *Photorhabdus* strains further defined the genus (Fischer-Le Saux et al., 1999). Using a broader definition of a species (60–80% relatedness,  $\Delta T_m$  between 5°C and 7°C divergence), Fischer-Le Saux et al. (1999) proposed the creation of two new nomenclatures. One species, designated “*P. asymbiotica*,” contained clinical isolates of *P. luminescens* previously recognized as a unique taxon by Akhurst and colleagues (Akhurst et al., 1996). The second new species, *P. temperata*, consisted of seven endosymbiotic strains isolated from *Heterorhabditis bacteriophora*, *Heterorhabditis megidis* or *Heterorhabditis zealandica*. Both *P. luminescens* and *P. temperata* could be further divided into subspecies, with strains in each subgroup exhibiting >80% relatedness with  $\Delta T_m$  of <1.5°C (Fischer-Le Saux et al., 1999); however, there are many taxonomic issues regarding *Photorhabdus*. The type strain (Hb) for *Photorhabdus* appears to be an atypical representative of the genus, since only one other strain belonging to the same taxa (*P. luminescens* subsp. *luminescens*) has been identified. Also the number of *Photorhabdus* strains isolated from diverse hosts needs to be expanded to better understand the genetic diversity and phylogenetic depth of the genus. Recent DNA relatedness studies clearly indicate that there are strains with unique 16S rDNA genotypes that do not reside within any current recognized *Photorhabdus* nomenclatures based upon DNA-DNA pairing studies (Fischer-Le Saux et al., 1998; Fischer-Le Saux et al., 1999). Finally, whether a relaxed species concept as proposed by Fischer-Le Saux et al. (1999) to delineate taxa within the genus *Photorhabdus* is relevant remains to be determined.

*Photorhabdus* is a member of the  $\gamma$  subdivision of the class Proteobacteria (Ehlers et al., 1988). Phylogenetic trees constructed using 16S rDNA sequence data places *Proteus vulgaris* and *Arsenophonus nasoniae* as its nearest neighbors exhibiting 93.5–95.1% sequence similarity (Rainey et al., 1995; Suzuki et al., 1996; Liu et al.

1997; Szállás et al., 1997; Spröer et al., 1999). The phylogenetic positioning of the genus next to *Proteus*, however, indicates that *Photorhabdus* is not an ancient (“core”) member of this family. Another association linking *Photorhabdus* with the family Enterobacteriaceae is the fact that *P. luminescens* ATCC 29999 and ATCC 29304 contain the 16S rRNA signature sequence CAAC-CCUUAUCCUUUG, which is only found in enterobacteria (Ehlers et al., 1988). *Xenorhabdus* species can be distinguished from *Photorhabdus* by the occurrence of a signature nucleotide sequence between positions 208 and 211 (*E. coli* numbering) of 16S rDNA (Szállás et al., 1997). *Photorhabdus luminescens* expresses the ECA (Ramia et al., 1982; Böttger et al., 1987). The G+C content of the genus is 43–44 mol% (Boemare et al., 1993).

*Photorhabdus* does not apparently exist in a free-living form in the environment since genetically marked strains of *Photorhabdus* released into river water or soil samples fail to survive for more than 7 days (Morgan et al., 1997). Rather *Photorhabdus* species appear to coexist in nature in an intimate symbiotic association with entomopathogenic nematodes of the family Heterorhabditidae. *Photorhabdus* bacteria live throughout the intestine of infective juvenile nematodes (third-instar, L3 stage, dauer larva) that are present in soil waiting to prey on their next insect host (Forst et al., 1997). Nematodes entering the larval stage of a variety of insects eventually penetrate into the hemocoel of the host, acting as a “living syringe” by releasing symbiotic bacteria into the hemolymph (Forst et al., 1997; Fischer-Le Saux et al., 1998). Acting in concert, bacteria and nematodes then kill insect larva with *Photorhabdus* species playing a major pathogenic role. Within the hemocoel of the larval carcass, nematodes reproduce sexually and bacteria grow to stationary phase (Forst et al., 1997). The nematode provides protection and transport for the bacterial endosymbionts in soil and in the insect host (Liu et al., 1997; Fischer-Le Saux et al., 1999). In return, bacteria symbionts provide nutrients for nematodes and release antimicrobial substances that suppress contamination of the cadaver by other microorganisms, thereby facilitating their sexual reproduction in the insect carcass (Forst and Neilson, 1996). Prior to release from this carcass, bacteria reassociate with nematodes that are then released as infective juveniles ready to seek a new host as the life cycle repeats itself. Up to 0.5 million infective nematodes per gram of host are released from the cadaver two or more weeks after death (Boemare and Akhurst, 1988).

An additional characteristic of the life cycle in entomopathogenic nematodes of *Photorhabdus* spp. is phase variation. Bacterial cells recovered

Table 22. Distinguishing features of phase I and II cells of *Photorhabdus* spp.

Trait	Phase I	Phase II
Colonial morphology	Mucoid	Not mucoid
Bioluminescence	+++	– or ↓↓↓
Antimicrobial activity against <i>Micrococcus luteus</i>	+++	– or ↓↓↓
Dye absorption (e.g., in MacConkey, of bromothymol blue, and Congo red)	+++	– or ↓↓↓
Production of lipase, phospholipase, and proteases	+++	– or ↓↓↓

Symbols: +++, strong activity; and – or ↓↓↓ no activity or reduced levels.

From Boemare and Akhurst (1988), Boemare et al. (1993), Akhurst et al. (1996), and Forst et al. (1997).

from infective juvenile nematodes are termed “phase I cells” or “primary form symbionts.” Upon *in vitro* cultivation or when directly isolated from clinical material, phase I cells convert to or exist as a more stable form called “phase II (secondary) cells” (Farmer et al., 1989; Forst et al., 1997). A number of phenetic differences exist between both phase variants and are listed in Table 22.

In addition to the properties described in Table 22, a number of other structural or phenotypic features distinguish phase I and II cells (reviewed in Forst et al., 1997). DNA pairing studies, however, clearly demonstrate that both phases are 100% related to each other in reciprocal hybridization experiments or in their relative binding ratios to *P. luminescens* Hb<sup>T</sup> (Boemare et al., 1993; Akhurst et al., 1996). Some intermediate-type colonies have been described that possess one or more phase I characteristics (Boemare et al., 1993).

The genus *Photorhabdus* consists of Gram-negative pleomorphic rods that range in size from 2 to 10  $\mu\text{m}$  by 0.5 to 2.0  $\mu\text{m}$  depending upon the age of the culture and phase of the isolate (see above); filaments as long as 30  $\mu\text{m}$  can occur (Boemare et al., 1993). Bacteria are motile via peritrichous flagellation. Many strains produce red, pink or yellow colonies on enriched media, although this is not an invariable characteristic (Boemare and Akhurst, 1988; Boemare et al., 1993; Peel et al., 1999). Cardinal features of *Photorhabdus* species useful in separating them from other Enterobacteriaceae include bioluminescence, a negative nitrate reductase reaction, and an unusual type of hemolysis (Fischer-Le Saux et al., 1999; Peel et al., 1999). This last attribute, which occurs on sheep blood agar, was originally described by Farmer and colleagues (Farmer et al., 1989) and consists of no hemolysis immediately surrounding the colony but rather a thin line (~2 mm) of hemolysis about 13 mm from the edge. This type of hemolysis is now referred to as “annular hemolysis” (Fischer-Le Saux et al., 1999). Consistent biochemical features of the genus include negative reactions for lysine and ornithine decarboxylase, arginine dihydrolase,

Table 23. Distinguishing features of *Photorhabdus* species.

Test <sup>a</sup>	<i>P. luminescens</i>	<i>P. temperata</i>	<i>P. asymbiotica</i>
Indole	+	– <sup>b</sup>	–
DNase	– <sup>c</sup>	+	–
Urease	V	– <sup>b</sup>	+
Citrate	+ <sup>c</sup>	+ <sup>b</sup>	+
Utilization of L-Fucose	+ <sup>c</sup>	+ <sup>b</sup>	–

Symbols: see footnote in Table 4.

<sup>a</sup>Reactions are at 28°C.

<sup>b</sup>Rare strains occur that deviate from the indicated phenotype.

<sup>c</sup>Except *P. luminescens* subsp. *laumondii*.

From Fischer-Le Saux et al. (1999).

and ONPG (*o*-nitrophenyl- $\beta$ -galactopyranoside) activity. Few carbohydrates are fermented with the exception of D-glucose, fructose and D-mannose. *Photorhabdus* species need to be separated from other H<sub>2</sub>S-negative genera expressing similar phenotypic traits (see Table 10). Comprehensive studies of the antimicrobial susceptibility profiles of clinical isolates of *Photorhabdus* (*P. asymbiotica*) have not been undertaken. From individual reports it appears that most strains are susceptible *in vitro* to the aminoglycosides, tetracycline, naldixic acid and third-generation cephalosporins, while often resistant to ampicillin, cephalothin and penicillin (Farmer et al., 1989; Peel et al., 1999). *In vitro* susceptibility results may not, however, always correlate with clinical response (Peel et al., 1999).

Biochemical tests useful in the identification of *Photorhabdus* spp. are listed in Table 23.

At present, the identification of *Photorhabdus* species can be fraught with many difficulties. Two genomospecies, *P. luminescens* and *P. temperata*, each contain one or more strains that do not taxonomically reside within described subspecies (Fischer-Le Saux et al., 1999). These strains often have phenotypic properties that are different from those described for named subspecies within each taxa. Farmer et al. (1989) have also indicated that the inactivity of *P. asymbiotica*

strains (formerly *X. luminescens* DNA hybridization group 5) may cause problems for clinical laboratories in regards to their recognition and identification.

The bioluminescence genes have been cloned from several strains of *P. luminescens* (strains Hm, Hb and HW) and expressed in *E. coli*. The organization of these strains is similar to those in other bioluminescent species with the luciferase genes (*luxA* and *luxB*) flanked by genes encoding for enzymes of the fatty acid reductase complex (Frackman et al., 1990). The order of genes in strain HW is CDABE (Xi et al., 1991). Probes constructed to *luxA* and *luxB*, which encode for the  $\alpha$  and  $\beta$  subunits of the luciferase gene, react in Southern blots against all *P. luminescens* strains including primary and secondary forms (Frackman et al., 1990). Under low stringency conditions, these probes also hybridize to other bioluminescent bacteria in the genera *Vibrio* and *Photobacterium*. The  $\alpha$  and  $\beta$  subunits of the luciferase gene of strain HW exhibit 51–83% sequence identity to those of *V. harveyi*, *V. fischeri* and *P. leiognathi* and 84–93% sequence identity to the *luxA* and *luxB* genes of other *P. luminescens* strains (Xi et al., 1991). A number of genes associated with the toxin complex loci (*tc*) of *P. luminescens* have been cloned (ffrench-Constant and Bowen, 1999; Waterfield et al., 2001). These four loci (*tca*, *tcb*, *tcc* and *tcd*) appear to demonstrate little homology to previously identified bacterial toxins and no significant overall similarity to any sequences deposited in GenBank (ffrench-Constant and Bowen, 1999). Their precise composition and mode of action are unknown.

Because of their ability to kill a variety of insects ranging from cockroaches to boll weevils, *Photorhabdus* species are viewed as potentially important bacteria useful in insect pest control (Forst et al., 1997). The use of *Photorhabdus* in insect pest control may have important advantages over other entomopathogenic bacteria such as *Bacillus thuringiensis* since the former species may have an expanded host range and may produce new pesticidal toxins to which insects have not acquired resistance. A toxin complex (*tc*) locus in *P. luminescens* encodes for four toxin complexes designated “Tca,” “Tcb,” “Tcc” and “Tcd” (Waterfield et al., 2001). The exact composition and function of these high molecular weight insecticidal toxin complexes is presently unknown, although a number of ORFs found around the *tc* complex exhibit similarity to *E. coli* colicins and *Pseudomonas aeruginosa* pyocin (Waterfield et al., 2001). In addition to their pathogenic role in insects, two recent reports have described the isolation of *Photorhabdus* strains from human infections (Farmer et al., 1989; Peel et al., 1999). Farmer and others

Table 24. Type strains of *Photorhabdus* species.

Species	Type strain	Other designations
<i>P. luminescens</i>	Hb	ATCC 29999; DSM 3368; NCIB 12670
<i>P. temperata</i>	X1Nach	CIP 105563
<i>P. asymbiotica</i>	CDC 3265-86	ATCC 43950

(Farmer et al., 1989) reported on six isolates (including one case in the addendum) of *Xenorhabdus luminescens* (now *P. asymbiotica*) that had been recovered from clinical specimens including wounds and blood. Although the medical history on three of these cases was fragmentary, there was compelling evidence in two instances (one involving a wound infection of the lower leg in a 45-year-old man) that *Photorhabdus* was indeed a human pathogen. A second case involved a 78-year-old man with a pretibial wound infection, which implicated *Photorhabdus* as a copathogen with *Staphylococcus aureus*. A decade later, Peel et al. (1999) described four additional illnesses linked to *Photorhabdus luminescens* infection. Three of these four illnesses involved middle-aged to elderly persons with multiple cutaneous lesions (one with a positive blood culture) that primarily affected the limbs. A fourth patient, a 90-year-old male, apparently developed *Photorhabdus* bacteremia of unknown etiology. One striking finding from both studies was a clinical history documenting a spider bite in two cases and a suggested bite in a third that preceded development of *Photorhabdus* wound infections. This indicates arachnids may be potential vehicles of *Photorhabdus* infections.

The type strains of *Photorhabdus* species are listed in Table 24.

## Pragia

*Pragia fontium* is the only species in the genus *Pragia* (Aldová et al., 1988; Brenner, 1991). Bouvet et al. (1985) first showed *P. fontium* was a different species than *B. aquatica* (both species are H<sub>2</sub>S-positive and are isolated from water). The 18 known *Pragia* strains were all isolated in Czechoslovakia, 17 from well water or water pipes and one from the stool of a healthy woman (Aldová et al., 1988; Brenner, 1991). Phylogenetic analysis of the type strain of *P. fontium* indicates that *Pragia* forms a distinct lineage in the family, well separated from other Enterobacteriaceae genera (Spröer et al., 1999).

*Pragia fontium* usually gives – + – + IMViC reactions, is H<sub>2</sub>S-positive, motile, and LDC-, ODC-, and ADH-negative (Brenner, 1991). It ferments very few sugars other than D-glucose

and D-galactose and does not grow at 42°C. Oxidation of gluconate by *P. fontium* helps to separate *Pragia* from *Budvicia* and other H<sub>2</sub>S-producing genera in the family Enterobacteriaceae (Aldová et al., 1988). Additional characteristics useful in separating H<sub>2</sub>S-positive genera/species can be found in Table 4. Whole cell protein profiles can also be used to distinguish *Pragia* from phenotypically similar genera including *Budvicia* and *Leminorella* as strains of each group cluster together (Schindler et al., 1992). Some strains produce a *Shigella*-like odor on nutrient agar (Aldová et al., 1988). An isolation medium has been designed by Schubert and Groeger-Söhn (1998) for the recovery of *P. fontium* from surface waters. This medium differs from that described for *B. aquatica* (see *Budvicia*) only in the concentration of cephalothin (15.0 µg/ml) added to the basal medium.

Seven strains of *P. fontium* were 85–94% related to the type strain (CNCTC Eb11/82) at both 60°C and 75°C using the hydroxapatite method. Among members of the Enterobacteriaceae, *P. fontium* was most closely related to *B. aquatica* (37%) but was 17% or less related to all other members of this family. The G+C content is 46–47 mol% (Aldová et al., 1988). *Pragia fontium* produces the ECA (Böttger et al., 1987).

The type strain of *P. fontium* is CNCTC Eb11/82 (= ATCC 49100 = CCUG 18073 = CDC 963-84 = DRL 20125 = IP 20125).

## Rahnella

Gavini et al. (1976), during a study of the genus *Enterobacter*, used the name “Group H<sub>2</sub>” for phenotypically similar, phenylalanine deaminase-positive strains that may have been included in the *Enterobacter agglomerans*-*Erwinia herbicola* complex on the basis of their negative lysine- and ornithine decarboxylase reactions, and negative arginine dihydrolase reaction (Brenner, 1991). On the basis of DNA relatedness reactions on 12 of the 20 strains of Group H<sub>2</sub>, a single species, *Rahnella aquatilis*, was proposed in the new genus *Rahnella* (Izard et al., 1979). Relatedness values to the labeled type strain were 57–98% with four of the 11 strains tested giving values below the 70% usually considered to reflect species-level relatedness (Brenner, 1991). Recent investigations employing a polyphasic taxonomic approach have identified at least three genomospecies within *Rahnella aquatilis* (Brenner et al., 1998). Genomospecies 1 includes the type strain of *Rahnella aquatilis* and 17 other strains. The mean DNA interspecies relatedness of genomospecies 1 strains (*R. aquatilis sensu stricto*) was 88% at

60°C (range 72–100%) with a mean divergence of 3.5% (range 0–7%). Genomospecies 2 consisted of 30 strains primarily from contaminated blood, snails and water. Interspecies relatedness was 93% (range 73–100%) with a mean sequence divergence of 1.5% (range 0.5–5.5%) in 60°C reactions. The final DNA group consists of a single strain (DSM 30078) from an unknown source. The type strain of *R. aquatilis* (CCUG 14185) was 70% related with 8% divergence to genomospecies 2 in 60°C reactions and 50% related in 75°C reactions. CCUG 14185 was 64% related with 9% divergence in 60°C reactions to DSM 30078 (Brenner et al., 1998). The reference strain for genomospecies 2 was 56% related with 8% divergence to strains belonging to *R. aquatilis* (genomospecies 1). Because genomospecies 2 and 3 could not be unambiguously separated phenotypically from *R. aquatilis sensu stricto*, they were left unnamed and the vernacular names “*Rahnella* genomospecies 2 and 3” were proposed (Brenner et al., 1998). Detailed phylogenetic investigations on the genus *Rahnella* have not been undertaken; however, two independent studies that have constructed dendrograms of the major members of the family Enterobacteriaceae using 16S rDNA data have identified *E. americana* as the closest neighbor of *R. aquatilis* (Hauben et al., 1998; Spröer et al., 1999). The G+C content of *R. aquatilis* and related organisms is 51–56 mol% (Izard et al., 1979; Brenner, 1991). *Rahnella aquatilis* produces the ECA (Böttger et al., 1987).

*Rahnella* is widely distributed in nature with water being the major reservoir for these microorganisms (Farmer et al., 1985). *Rahnella aquatilis* has also been recovered from a variety of other environmental niches, including soil, the rhizosphere of wheat and maize, from fermenting wort, and from fresh Italian ryegrass (Berge et al., 1991; Hamze et al., 1991; Heron et al., 1993). It has even been recently identified from the intestinal contents of a 12,000-year-old mastodon (*Mammuth americanum*) using 16S ribosomal (rDNA) sequencing (Rhodes et al., 1998). Many food products also contain *R. aquatilis* such as vegetables (Österblad et al., 1999). A Swedish study of minced meat, retail fish, and pasteurized milk and cream yielded both *R. aquatilis* and *Hafnia alvei* in high numbers (>10<sup>6</sup> cfu/g) in all three products (Lindberg et al., 1998). In addition to environmental foci, *R. aquatilis* has been recovered from human blood, urine and respiratory secretions (Oh and Tay, 1995).

*Rahnella aquatilis* is a nonpigmented bacillus that is motile at 25°C, but not at 36°C (Brenner, 1991). Strains of *Rahnella aquatilis* deaminate phenylalanine, but the reaction is weaker than seen in *Proteus* and *Providencia* (Farmer et al.,

1985). The ONPG reaction is positive. *Rahnella aquatilis* is reported to be negative for H<sub>2</sub>S, lysine and ornithine decarboxylase, and arginine dihydrolase by Farmer et al. (1985) and later Brenner et al. (1998) and thus must be separated from phenotypically similar organisms (see Table 10). However, the original description states that the arginine dihydrolase reaction is variable, with most strains positive (Izard et al., 1979). Variation in ornithine decarboxylase reactions has also been reported by different laboratories (Brenner et al., 1998). Most *Rahnella* and *Rahnella*-like strains ferment L-arabinose, D-glucose, lactose, D-mannitol, salicin, cellobiose, maltose, D-mannose, melibiose, L-rhamnose and sucrose (Brenner et al., 1998). Genomespecies 1 (*R. aquatilis sensu stricto*) and genomespecies 2 cannot be unambiguously differentiated using phenotypic methods, although utilization of L-histidine, L-ornithine, L-proline and hydrolysis of *para*-nitrophenyl- $\beta$ -D-glucopyranoside and L-proline-*para*-nitroanilide are useful in distinguishing these two genomespecies (Brenner et al., 1998). Most *R. aquatilis* and *R. aquatilis*-like strains are resistant to aminopenicillins (e.g., ampicillin and amoxicillin), first generation cephalosporins and cephamycins (Funke and Rosner, 1995; Stock et al., 2000). Resistance to second-generation cephalosporins, such as cefuroxime, has also been noted.

*Rahnella aquatilis* is an uncommon human pathogen. Most human infections have involved episodes of bacteremia in men (less commonly in women) that are either immunocompromised or have other serious underlying illnesses (Oh and Tay, 1995; Reina and Lopez, 1996); however, cases of *R. aquatilis* sepsis in immunocompetent adults have been described (Chang et al., 1999). Nosocomial transmission of *R. aquatilis* has also been documented on one occasion (Caroff et al., 1998). Other *R. aquatilis*-related diseases include urinary tract infections, postsurgical wound infections, and endocarditis (Alballaa et al., 1992; Maraki et al., 1994; Matsukura et al., 1996). Although the sources for most human illnesses remain unknown, both Hickman catheters and contaminated parenteral nutrition have been implicated as vehicles of infection (Oh and Tay, 1995; Caroff et al., 1998). In one instance, *R. aquatilis* sepsis resulted from a contaminated (1000-ml bottle of) 5% dextrose-vitamin B complex-vitamin C solution bought over-the-counter by a 26-year-old Korean man who was subsequently infused by an unlicensed individual (Chang et al., 1999). Most strains are susceptible to the aminoglycosides, third generation cephalosporins, quinolones, carbapenems, monobactams, tetracyclines and sulfa-containing compounds (Funke and Rosner, 1995; Caroff et al., 1998; Stock et al., 2000).

The type strain of *R. aquatilis* is 133 (= CIP 78-65 = ATCC 33071).

## *Raoultella*

Drancourt et al. (2001) have proposed the transfer of three *Klebsiella* species to a new genus, *Raoultella*, based upon phylogenetic analyses of 16S rDNA and *rpoB* (bacterial RNA polymerase  $\beta$ -subunit) genes. Using neighbor-joining and maximum-parsimony methods, three clusters within *Klebsiella* were evident with cluster II containing *K. ornithinolytica*, *K. terrigena* and *K. planticola*. These three species share several phenotypic traits in common with each other including growth at 10°C and utilization of L-sorbose as a carbon source. Using 98% 16S rDNA and 94% *rpoB* sequence similarity as breakpoints for delineating different genera, the proposal to transfer these three species to the genus *Raoultella* was made (Drancourt et al., 2001); however, these three species do not form as tight a phyletic line as cluster I, which contains the three subspecies of *K. pneumoniae* with 98.2–99.7% 16S rDNA sequence similarity and 99.4–100% *rpoB* sequence similarity, respectively. Additionally, the placement of *K. terrigena* in this genus is questionable and further DNA pairing studies seem warranted.

## *Sodalis*

Members of the genus *Sodalis* are secondary (S) endosymbionts of the tsetse fly, *Glossina morsitans morsitans*, and reside in multiple host tissues, including midgut epithelial cells (Dale and Maudlin, 1999). Infection of insect cells in vitro appears to be facilitated through *Sodalis* genes that encode for components of a type III secretory system, which include a gene with a high sequence identity to *invC* (Dale et al., 2001b). Previous studies on S endosymbionts have demonstrated they are members of the  $\gamma$ -3 subclass of the class Proteobacteria and form a distinct lineage within the family Enterobacteriaceae (Aksoy et al., 1995b; Dale and Maudlin, 1999). In 1999, Dale and Maudlin were able to successfully isolate and grow an axenic culture (strain M1) of an S endosymbiont from a previously infected insect cell line of *Aedes albopictus* inoculated with hemolymph from a laboratory colony of tsetse flies. Based upon phylogenetic analysis of these organisms and unique phenotypic properties associated with agar-grown bacteria, the name "*Sodalis glossinidius*" was proposed to include S-endosymbionts found in *G. morsitans morsitans* and *G. pallidipes* (Dale and Maudlin, 1999).



*Sodalis* appears to be vertically transmitted through maternal milk glands (Cheng and Aksoy, 1999). Eradication of S-endosymbionts (*S. glossinidius*) from tsetse flies with streptozotocin has little effect upon fly reproductivity but does reduce longevity (Dale and Welburn, 2001a). Eradication also increases the refractory nature of tsetse flies to parasitism by trypanosomes. This suggests S-endosymbionts might serve as important delivery systems for anti-parasite gene expression (Dale and Maudlin, 1999). *Sodalis glossinidius* can either be cultured using mosquito (*Aedes albopictus*) feeder cell culture systems or can be grown in vitro on Mitsuhashi-Maramorosch (MM) agar supplemented with fresh horse blood or catalase (Dale and Maudlin, 1999). Optimal growth occurs under microaerophilic conditions (<10% oxygen) at 25°C; no growth occurs at temperatures exceeding 30°C. Colonial morphology is pleomorphic and not maintained upon subculture. Cells are nonmotile, filamentous, Gram-negative rods, 2–12 µm in length and 1–1.5 µm in diameter (Dale and Maudlin, 1999). *Sodalis glossinidius* is catalase-negative, an unusual trait for members of the family Enterobacteriaceae. Metabolically, *Sodalis* is very inactive with few carbohydrates fermented or assimilated. Exceptions to this rule include D-glucose, D-mannitol, sorbitol, raffinose, N-acetyl-D-glucosamine, and glycol chitosan (Dale and Maudlin, 1999). *Sodalis glossinidius* produces at least one type of chitinase (Dale and Maudlin, 1999; Dale and Welburn, 2001a).

The type strain of *S. glossinidius* is M1 (= NCIMB 13495).

## *Tatumella*

*Tatumella ptyseos*, the only species in *Tatumella*, was proposed by Hollis et al. (1981) for strains formerly called “EF-9” (eugonic fermenter; eugonic means rapid and relatively luxuriant growth). DNA relatedness of 25 strains to the type strain was 80–100% at an optimal reassociation temperature and 68–100% at a stringent reassociation temperature (Hollis et al., 1981; Brenner, 1991). Divergence within related sequences was 0.5% or less for 19 strains, but was 5.5–8.0% for 6 others (Brenner, 1991). Such high divergence values are extremely unusual within strains of a species; however, since these strains exhibited species-level relatedness to the type strain (as high as 88%) and were biochemically typical, they were included in the species. *Tatumella ptyseos* was less than 40% related to other Enterobacteriaceae and was 2% related to the type strain of *Chromobacterium violaceum* (Brenner, 1991). Phylogenetic analysis of the

type strain of *T. ptyseos* indicates that *Tatumella* forms a distinct lineage in the family well separated from other Enterobacteriaceae genera (Spröer et al., 1999). The G+C content of *T. ptyseos* is 53–54 mol%. Diaminopropane and putrescine are the major polyamines produced (Hamana, 1996). *Tatumella ptyseos* produces ECA (Ramia et al., 1982).

Over fifty *T. ptyseos* strains are now in the CDC collection (Farmer et al., 1985). Forty-six of these are from the respiratory tract (mainly sputum), which is reflected in the species name *ptyseos*, which means “a spitting.” Three isolates are from blood. *Tatumella ptyseos* has additionally been recovered from the blood of a Malaysian neonate with jaundice and presumed sepsis (Tan et al., 1989). She was treated with ampicillin and gentamicin and recovered uneventfully.

*Tatumella ptyseos* is an H<sub>2</sub>S-negative species that is lysine and ornithine decarboxylase- and arginine dihydrolase-negative. Thus, it must be separated from phenotypically similar genera in the Enterobacteriaceae (Table 10). It is also indole- and Voges-Proskauer-negative and ferments few carbohydrates other than D-glucose, sucrose, trehalose and salicin (some strains). *Tatumella ptyseos* is quite atypical for Enterobacteriaceae in several phenotypic characteristics (Brenner, 1991). It is generally more active at 25°C and is nonmotile at 36°C. Two-thirds of strains are motile at 25°C. Flagella are difficult to demonstrate, and when present, are polar, subpolar, or lateral—not peritrichous as in all other motile members of the family (Hollis et al., 1981; Brenner, 1991). Strains are susceptible to penicillin. All strains grow on MacConkey and other plating media commonly used for Enterobacteriaceae (Brenner, 1991). Survival on commonly used agars or semisolid stock culture media is not always good, and *T. ptyseos* does not survive for a week on blood agar. It is recommended that the organism be held frozen at –40°C or colder in 5% defibrinated rabbit blood for long-term storage, although this process may not ensure preservation of all strains (Hollis et al., 1981).

The type strain of *T. ptyseos* is D16168 (= CDC 9591-78 = ATCC 33301 = DSM 5000).

## *Trabulsiella*

The genus *Trabulsiella* consists of a single species, *T. guamensis*, previously referred to by the vernacular name “CDC Enteric Group 90” (McWhorter et al., 1991). Seven strains were found to be 98–100% related to the type strain at 60°C and 94–100% related at 75°C. The type strain of *T. guamensis* was 6–41% related to 62 other strains from other Enterobacteriaceae spe-

cies. The highest relatedness values obtained from DNA pairing studies were with *Salmonella* and *Kluyvera ascorbata* (McWhorter et al., 1991).

In the original description of the genus, McWhorter et al. (1991) described eight strains, six of which were isolated from vacuum cleaner dust in Guam. The remaining *T. guamensis* strains originated from human stool specimens from Germany and New York. An addendum to this article reported on four additional strains. Three of these strains were from environmental sources (one from wheat flour and two were unspecified) and a fourth strain was from the feces of a 44-year-old German man with a fever of unknown origin (McWhorter et al., 1991). There is no evidence presently that *T. guamensis* causes disease in humans.

Strains of *Trabulsiella* were originally recognized because of their biochemical similarity to *Salmonella* subgroups 4 and 5, although the former group failed to agglutinate in *Salmonella* O and H typing sera (McWhorter et al., 1991). *Trabulsiella guamensis* is a motile, oxidase-negative, facultatively anaerobic Gram-negative rod that produces H<sub>2</sub>S on triple sugar iron and peptone iron agar slants (Table 4). It produces both lysine and ornithine decarboxylase within 24 hours; 50% of strains also produce arginine dihydrolase within 48 hours. *Trabulsiella* is indole- and Voges-Proskauer-negative, ONPG- and mucate-positive, and produces acid from the fermentation of a large number of carbohydrates including D-glucose, L-arabinose, cellobiose, D-mannitol, L-rhamnose and D-sorbitol. There appear to be at least two biogroups of *T. guamensis*. Biogroup 1 strains are indole-positive, and hydrolyze gelatin (film method) and esculin. Biogroup 2 strains are negative for these traits. An unusual biochemical property of the genus is the ability to hydrolyze tyrosine crystals (McWhorter et al., 1991). *Trabulsiella guamensis* is susceptible to gentamicin, chloramphenicol, sulfamethoxazole, naladixic acid and resistant to penicillin, ampicillin and cephalothin by disk diffusion (McWhorter et al., 1991).

The type strain of *T. guamensis* is CDC 0370-85 (= ATCC 49490 = CIP 103637).

## Wigglesworthia

The genus *Wigglesworthia* consists of a single species, *W. glossinidia*, that is a primary (P) endosymbiont of tsetse flies belonging to species and subspecies of the genus *Glossina* (Aksoy, 1995a). Unlike S endosymbionts (see *Sodalis*), *W. glossinidia* resides in specialized cells called “mycetocytes” that are located in the anterior region of the gut. These specialized cells form a white

U-shaped organelle called a “mycetome” (Aksoy, 1995a). *Wigglesworthia glossinidia* contains only a single operon encoding for 16S rDNA sequences. Phylogenetic analysis of 16S rDNA and ribosomal DNA internal transcribed spacer-2 (ITS-2) from P endosymbionts indicates that *Wigglesworthia* forms a distinct lineage in the  $\gamma$  subdivision of the Proteobacteria and exhibits concordant evolution with its insect host (Aksoy et al., 1997; Chen et al., 1999). Based upon 16S rDNA data, *W. glossinidia* is most closely related to the genus *Buchnera*, to S-endosymbionts of other insects, and to *Escherichia coli* (Aksoy, 1995a).

The exact mechanism by which maternal transmission of *Wigglesworthia* occurs is presently unknown (Aksoy et al., 1997). Eradication of P endosymbionts through treatment of tsetse flies with ampicillin and tetracycline results in a greater than 50% reduction in fecundity and pupal emergence, which essentially leads to insect sterility (Dale and Welburn, 2001a).

*Wigglesworthia glossinidia* cannot be grown outside the insect host, although it can be metabolically maintained active under transient tissue culture conditions for 72 hours or more (Aksoy, 1995a). Bacterial cells within mycetomes are rod-shaped and 4–5  $\mu$ m in length.

There is no type strain for *Wigglesworthia*. The genus and species were proposed on the basis of Rule 16a of the *International Code of Nomenclature of Bacteria* (Aksoy, 1995a).

## Xenorhabdus

In 1965, Poinar and Thomas proposed the name “*Achromobacter nematophilus*” for the symbiotic organism found in the digestive tract of the nematode *Steinernema* (formerly *Neoplectana*) *feltiae*. The genus name was subsequently rejected, resulting in a lengthy chronology of taxonomic proposals naming the bacterial symbiont and its nematode host (for a review, see Forst and Neilson, 1996). In 1979, Thomas and Poinar proposed the new genus *Xenorhabdus* in the family Enterobacteriaceae for the nematode symbiotic bacteria (Brenner, 1991). The genus was originally composed of two species, which included the type species, *X. nematophilus*, and the bioluminescent organism *X. luminescens*. The later species was subsequently transferred to the genus *Photorhabdus* as *P. luminescens* (Boemare et al., 1993; see *Photorhabdus*).

Akhurst (1983) proposed three subspecies of *X. nematophilus* on the basis of nematode host, pigment, maximum growth temperature, and biochemical tests (Brenner, 1991). These subspecies were designated “*X. nematophilus* subsp. *nematophilus*”, “*X. nematophilus* subsp. *bovie-*

nii” and “*X. nematophilus* subsp. *poinarii*.” He later described a fourth subspecies, “*X. nematophilus* subsp. *beddingii*,” for strains symbiotic with two undescribed *Steinernema* species (Brenner, 1991). Akhurst and Boemare (1988) proposed elevating of all of these subspecies to species status based upon numerical taxonomy studies involving 60 variable characters associated with *Xenorhabdus* subspecies and their associations with specific nematode hosts. Boemare et al. (1993) subsequently studied a broad sample of *Xenorhabdus* isolates by DNA hybridization. Using the hydroxyapatite method, *P.* (“*Xenorhabdus*”) *luminescens* was found to be only 4–11% related to other *Xenorhabdus* species (see *Photorhabdus*) and 0–15% related to 73 species of Enterobacteriaceae (Grimont et al., 1984; Boemare et al., 1993). Interspecies relatedness for *Xenorhabdus* (other than *P. luminescens*) was greater than 63%, while intraspecies relatedness ranged from 17 to 38%. These results confirm the validity of the proposed *Xenorhabdus* species as suggested by Akhurst and Boemare (1988). A fifth symbiont species, *X. japonicus*, associated with the nematode *Steinernema kushidai*, was described by Nishimura et al. (1994). The type strain (SK-1) was 29–51% related to other *Xenorhabdus* species in DNA-DNA hybridization assays.

*Xenorhabdus* is a member of the  $\gamma$  subdivision of the class Proteobacteria and forms a distinct side branch from core genera of the family Enterobacteriaceae (Ehlers et al., 1988; Stackebrandt et al., 1988). Binary similarity coefficients (0.65–0.69) of rRNA catalogues link the Enterobacteriaceae as nearest neighbors to *Xenorhabdus* and *Photorhabdus* when compared to the Aeromonadaceae (0.58), *Pasteurella* (0.53), vibrios and other marine organisms (0.35–0.55). They also contain several signature oligonucleotides present in all members of the family Enterobacteriaceae (Ehlers et al., 1988). On the intragenic level, *Xenorhabdus* species exhibit 97.2% 16S rDNA similarity and 92.7–96.1% similarity to other Enterobacteriaceae (Suzuki et al., 1996). Other groups of this subclass are less closely related with similarity values of less than 90% (Rainey et al., 1995). *Xenorhabdus* strains can be clearly distinguished from *P. luminescens* by a signature UUCG sequence present in the former genus between positions 208 to 211, while the latter group contains a longer UGAAAG version (Szállás et al., 1997); however, some controversy still exists regarding whether *Xenorhabdus* (as well as *Photorhabdus*) should be placed in this family (Forst and Neilson, 1996). This point of view is supported by fatty acid analyses and the fact that *Xenorhabdus* is unable to reduce nitrates and is catalase-negative, defining traits of this family. Equally controversial is the

phylogenetic position of both *Xenorhabdus* and *Photorhabdus*. While some distance matrices support the separate status of both entomopathogenic genera (Szállás et al., 1997; Forst et al., 1997), other 16S rDNA sequencing studies do not unambiguously come to the same conclusion (Rainey et al., 1995; Suzuki et al., 1996; Liu et al., 1997). The G+C content of *Xenorhabdus* is reportedly 43–50 mol% (Brenner, 1991). *Xenorhabdus nematophilus* produces the ECA (Ramia et al., 1982; Böttger et al., 1987).

*Xenorhabdus* is carried monoxenically in the intestine of juvenile, free-living, nonfeeding nematodes that are insect pathogens, or entomopathogens (Brenner, 1991). *Xenorhabdus nematophilus* contains several ORFs with extensive homology to the *tc* genes of *Photorhabdus*, suggesting that these sequences may have similar insecticidal activity to those found in *P. luminescens* (French-Constant and Brown, 1999). However, *X. poinari* is not pathogenic for common wax moth larvae (*Galleria mellonella*) and *X. japonicus* is not pathogenic for tobacco cutworm larvae (*Spodoptera litura*). Like *Photorhabdus*, *Xenorhabdus* species are symbionts of entomopathogenic nematodes, can be grown as free-living organisms in vitro, and display phenotypic variant forms (Forst and Neilson, 1996; Forst et al., 1997); however, they differ in several key aspects from one another, including nematode hosts (Table 25). Additional distinguishing characteristics can be found in the review of Forst and Neilson (1996).

*Xenorhabdus* has not been isolated in free-living form from soil or water and does not survive for more than 7 days in soil or water when introduced under experimental conditions (Morgan et al., 1997). This suggests that they require a symbiotic existence to survive in nature.

*Xenorhabdus* grows poorly and sometimes not at all at 36°C (Brenner, 1991). *Xenorhabdus* species do not reduce nitrates and are catalase-negative, reactions that are atypical for most members of the family Enterobacteriaceae (Brenner, 1991). They produce two colony forms, designated “phase I” and “phase II,” which are

Table 25. Selected distinguishing features of *Xenorhabdus* and *Photorhabdus*.

Character	<i>Xenorhabdus</i>	<i>Photorhabdus</i>
Nematode host	<i>Steinernema</i> spp.	<i>Heterorhabditis</i> spp.
Nematode location	Specialized vesicles	Present throughout gut
Bioluminescence	No	Yes
Catalase	No	Yes

Data from Forst and Neilson (1996) and Forst et al. (1997).

Table 26. Biochemical differentiation of *Xenorhabdus* species.

Test	<i>X. beddingii</i>	<i>X. bovienii</i>	<i>X. japonicus</i>	<i>X. nematophilus</i>	<i>X. poinarii</i>
Pigment on nutrient agar	B	Y	YB	U	R
Adsorption of bromothymol blue	+	+	+	+	–
Phosphatase	+	–	–	–	–
Esculin hydrolysis	+	–	–	–	V
Salicin, acid	+	–	ND	–	–
Lipase (Tween 80) <sup>a</sup>	–	+	–	–	+
Phenylalanine deaminase <sup>b</sup>	–	–	–	+	–
Assimilation of inosine, L-proline	+	+	–	+	+

Symbols and abbreviations: see footnote in Table 4; ND, not determined; B, brown; Y, yellow; YB, yellowish-brown; R, red; U, buff.

<sup>a</sup>Colony form 1 only.

<sup>b</sup>Colony form 2 only.

Adapted from Brenner (1991) and Nishimura et al. (1994).

Table 27. Type strains of *Xenorhabdus* species.

Species	Type strain	Other designations
<i>X. beddingii</i>	Q58	UQM 2871 (colony form 1); UQM 2872 (colony form 2); DSM 4764
<i>X. bovienii</i>	T228	UQM 2210 [ATCC 35271] (colony form 1); UQM 2211 [ATCC 49109] (colony form 2)
<i>X. japonicus</i>	SK-1	IAM 14265
<i>X. nematophilus</i>	ATCC 19061	
<i>X. poinarii</i>	G1	ATCC 35272; UQM 2216; DSM 4768

found in infective juveniles and as stable in vitro forms, respectively. These two phases exhibit similar enzymatic and antimicrobial activities to those found in *P. luminescens* with the exception that lipase activity is stronger in phase II cells and weaker in phase I variant forms (Forst and Neilson, 1996). Tests of value in differentiating *Xenorhabdus* species are shown in Table 26 (Brenner, 1991).

The type strains of *Xenorhabdus* species are listed in Table 27.

## *Yokenella* (*Koserella*)

In 1985, Hickman-Brenner et al. proposed the name “*Koserella trabulsii*” for a biochemical and phylogenetically new taxa that had been previously referred to by a number of vernacular names including “*Hafnia* species 3” and “CDC Enteric Group 45” (Farmer et al., 1985; Brenner, 1991). Kosako et al. (1984) independently concluded that a group of strains resembling *H. alvei*, called “NIH (Japan) biogroup 9,” was a single DNA relatedness group that they named “*Yokenella regensburgei*.” These two groups were subsequently found to be identical in a collaborative study (Kosako et al., 1987) and because *K. trabulsii* is a junior subjective synonym (published later) of *Y. regensburgei*, the latter has priority (standing) in nomenclature (Kosako and Sakazaki, 1991). For a more detailed description of the taxonomic contro-

versy surrounding *Yokenella* and *Koserella*, consult the review of Brenner (1991).

The NIH (Japan) and CDC collections contained 23 *Y. regensburgei* stains, 16 of which were isolated from humans in the United States and Japan (Brenner, 1991). Eight of these isolates were from wounds, three each from urine and sputum, one from stool, and one from an unknown source. Six strains were isolated from insect intestines collected in Germany and one from well water in the United States (Brenner, 1991). The role of this bacterium in human disease is unknown. However, two cases of possible *Y. regensburgei* infection have been described. One involved a wound isolate associated with a septic knee process in a 74-year-old man, while the other was a case of transient bacteremia in a 35-year-old woman with liver disease. These cases suggest that *Y. regensburgei* may have a limited pathogenicity in immunocompromised persons (Abbott and Janda, 1994).

*Yokenella regensburgei* has a – + – + IMViC pattern. It is positive for lysine and ornithine decarboxylase and most strains fail to produce arginine dihydrolase activity (Abbott and Janda, 1994; Farmer, 1999). *Yokenella regensburgei* tests negative for H<sub>2</sub>S production, urea, and phenylalanine deaminase (Brenner, 1991). Acid is produced from the fermentation of many carbohydrates including D-glucose, L-arabinose, cellobiose, D-mannitol, melibiose, L-rhamnose and D-xylose (Farmer, 1999).

The type strain of *Y. regensburgei* is NIH 725-83 (= JCM 2403).

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# Phylogenetic Relationships of Bacteria with Special Reference to Endosymbionts and Enteric Species

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## Introduction

The latter half of the 20<sup>th</sup> century saw two developments that revolutionized the study of bacterial systematics and our conceptions about microbial diversity. The first was the use of molecular genetic information, as gained through the analysis of protein and nucleic acid sequences, for the identification, typing and classification of microorganisms. The second was the application of the polymerase chain reaction (PCR) for the recovery of DNA sequences from noncultivable organisms.

By decoupling cultivation and classification, and by providing a common yardstick by which all organisms could be compared (i.e., small subunit ribosomal DNA sequences; hereafter referred to as “SSU rRNA”), these procedures added a new dimension to our knowledge of the microbial world. It is no longer necessary to test organisms against a panel of known phenotypic or biochemical properties, which, in themselves, are often variable in occurrence and can lead to cases of misclassification, or carry a preconceived notion about the characters that define a particular taxonomic group.

Despite the considerable advances made from applying these innovations, such an approach is not without its limitations. Characterization of a single conserved sequence, while often adequate for taxonomic purposes, does not reveal very much about the evolutionary history or biology of the organism *per se*. Whereas the placement of an organism (i.e., sequence) into an evolutionary framework may prompt numerous predictions about its morphology, ecology, physiology and biochemistry, virtually nothing can be said about its unique biology without cultivation or additional sequence information.

Other problems can arise when attempting to classify bacteria and to determine their relationships. These derive from two sources: the specific characters used to represent an organism (the choice of data) and the algorithms used to infer relationships (the methods used to construct the phylogeny or tree). With this in mind, genotypic

data hold several advantages over phenotypic characters for phylogenetic reconstruction in that: 1) variation in molecular sequences is discrete and well-defined; 2) molecular sequences comprise hundreds, if not thousands, of characters bearing potentially useful information; 3) molecular sequences accumulate informative changes, even when the resulting phenotypes are conserved; 4) informational macromolecules (DNA and proteins) change according to well-defined models of sequence evolution; and 5) identical phenotypes can arise by very different genetic mechanisms or pathways, such that changes other than those attributable to common ancestry can lead to the same phenotype.

Questions of character convergence are not limited to phenotypic traits: portions of molecular sequences may be identical owing to chance, natural selection, or lateral gene transfer. Furthermore, because the analysis of relatively small regions of DNA can furnish so much information, phylogenetic relationships are often based on data collected from a very limited portion of the genome, which may or may not be representative of the organism as a whole. Such problems stress the need to examine multiple genes and to infer statistically robust phylogenetic trees, which assists in recognizing inconsistencies in the data and in establishing an evolutionary framework for studying the biology of an organism.

## A Genomic View of Bacterial Evolution

The first goal of this chapter is to examine how information coming from completely sequenced microbial genomes has contributed to our interpretations of bacterial phylogeny and evolution. The molecular evolutionary history of microbes has largely been based on the evaluation of SSU rRNA (Fox et al., 1977; Fox et al., 1980; Woese, 1987; Hugenholtz et al., 1998; Maidak et al., 2000); however, the analysis of several conserved protein-coding regions has offered alternative scenarios relating to the branching orders of bac-

terial taxa, the rooting of the universal tree of life, and the role of lateral transfer in the early evolution and formation of genomes (Gogarten et al., 1989; Iwabe et al., 1989; Hilario and Gogarten, 1993; Brown and Doolittle, 1995; Golding and Gupta, 1995; Baldauf et al., 1996; Eisen, 1998; Forterre and Phillipe, 1999; Schutz et al., 2000). Such results have promoted the view that SSU rRNA might not fully reveal the relationships among organisms because genomes contain genes from multiple sources. Quite apart from the fact that SSU rRNA spans a very small portion and may not be representative of an entire genome, other concerns have been raised about the use of this molecule as the sole basis for molecular phylogenetic reconstructions. The size of SSU rRNA (ca.1600 nucleotides) and its very slow rate of evolution contribute to the fact that there are often too few variable sites to resolve phylogenetic relationships accurately. Furthermore, because rRNA does not encode a protein, the occurrence of frequent additions and deletions in certain portions of the molecule presents problems for sequence alignments, and selection of secondary structures can cause sequence convergence and saturation (homoplasy), thereby distorting the actual relationships among organisms (Hillis and Dixon, 1991; Kjer, 1995; Huynen et al., 1997).

Complete genomes allow immediate access to the full set of sequences that are conserved among diverse genomes, which has greatly facilitated the analysis of genes other than those encoding SSU rRNA. Perhaps a hundred genes are conserved among all bacterial lineages, affording the opportunity to trace the evolutionary history of sequences representing a substantial fraction of the genome. Furthermore, the availability of complete genome sequences has led to the development of numerous “whole-genome” approaches for evaluating the relationships among organisms. These studies are certainly among the most interesting endeavors in the analysis of bacterial genomes and have broadened the scope of evolutionary studies to include new levels of genetic organization.

The second portion in this chapter concerns the phylogenetic relationships among microorganisms classified as Enterobacteriaceae. Considering that this family of bacteria includes the workhorses of molecular, genetic, and biochemical analyses (*Escherichia coli* and *Salmonella*) and contains pathogens causing diseases with substantial impact on human populations (plague, typhoid and dysentery), there is perhaps more known about the biology of these microbes than for any other group of organisms. As in many other taxa, there has been a shift towards the use of molecular characters to infer the relationships among enterics; however, phenotypic

markers still form much of the basis for typing the majority of strains, particularly in clinical settings. Because most enteric species were originally differentiated on the basis of biotyping rather than on genotypic characters, several of the current designations are arbitrary and do not reflect either the extent of genetic diversity or the true relationships among strains.

As an example, the Shigellae have traditionally been subdivided into four species—*Shigella boydii*, *S. flexneri*, *S. dysenteriae* and *S. sonnei*—although the total amount of genetic variation within this genus is fully contained within *E. coli*. Therefore, *Shigella* should not be classified as a genus separate from *Escherichia*. Furthermore, the amount of genetic diversity varies widely among the four *Shigella* species: *S. sonnei* constitutes a single genetically uniform lineage, whereas each of the other three species comprises a very heterogeneous array of clones. Finally, the classification of *Shigella* strains does not reflect their genetic similarities: strains of *S. flexneri* can be more closely related to *S. boydii* than to any other strains typed as *S. flexneri*, and both *Shigella boydii* and *S. dysenteriae* have multiple independent (polyphyletic) origins from within *E. coli*. Clearly, the Shigellae warrant reclassification based on genetic criteria, although the epidemiological consequences of a taxonomic revision are uncertain.

Although there are numerous cases where traditional classification is at odds with information from molecular sequences, or where the phylogenetic relationships based on different molecular characters are in conflict, our purpose in this chapter is not to amend or revise the taxonomic nomenclature either of the Enterobacteriaceae or of the major phyla of bacteria. Rather, we outline the rationale of the phylogenetic approach as it applies to bacteria, describe several of the molecular characters and methods used to infer phylogenetic trees, and review the phylogenetic relationships of strains derived from these procedures. Hopefully, in doing so, we have accomplished our ultimate goal, which is to provide an evolutionary framework for understanding the remarkable diversity of bacteria.

## Bacterial Relationships and Realities

The living world is organized taxonomically into three domains—Archaea, Bacteria and Eucarya—which have been delineated in terms of their SSU rRNA sequences and cell membrane structures (Woese et al., 1990; Winker and Woese, 1991). Despite the prevailing nomenclature, Bacteria are the most ancient and are estimated to have appeared between 3.5 and 4

billion years ago. Bacteria comprise about 40 phyla (i.e., deep-branching subdivisions), about one-third of which presently contain representatives among the fully sequenced genomes. Naturally, cultivable, pathogenic, and small-genome bacteria have been the focus of most sequencing endeavors; however, substantial efforts are being made to expand both the phylogenetic and ecological scope of the set of sequenced microbes.

For assessing the broad-scale evolutionary relationships among the Bacteria, this chapter considers primarily those microbes for which full genome sequences are available. Although this limitation overlooks large portions of the genomic diversity within the Bacteria, it is useful to note that: 1) many recognized bacterial phyla are represented by only one or a few organisms for which we have little more than SSU rRNA sequences; and 2) the early radiation of the Bacteria into phyla occurred relatively rapidly, such that the set of sequenced organisms probably encompasses the range of variation within this domain.

The field of phylogenetics is constantly being challenged with new types of data, larger samples of organisms, and alternative models of evolution, and thus relies upon the continuous development of new methods to estimate, infer, test and resolve the relationships among organisms. Virtually all phylogenetic trees encountered in the literature are based on one of the following methods: 1) "Parsimony" finds the tree that requires the minimum number of changes; 2) "simple distance" generates the tree that best represents the matrix of pairwise distances between organisms; 3) "neighbor joining" is a distance method that progressively clusters pairs of taxa that yield the shortest total branch length; 4) "maximum likelihood" uncovers the tree that best fits a specified probabilistic model of sequence evolution; and 5) "Bayesian inference" relies upon the posterior probabilities of a tree to test the hypothesis that the particular topology is correct. Because the parsimony and likelihood methods compare all possible trees to find the optimal branching order, both become computationally intensive with increasing sample sizes. "Bootstrapping" is a data re-sampling procedure designed to test the degree of support for the branching order in a given tree, with numbers (bootstrap values) placed at nodes to indicate the percentage of replicates in which the same taxa are joined at a particular node.

## Sequence-alignment-based Bacterial Phylogenies

Since the early 1980s, when small-scale DNA sequencing became affordable, bacterial phylo-

genetics has relied upon the comparison of homologous sequences in different taxa and in particular on SSU rRNA. Such comparisons have allowed for an ability to comprehend the diversity within the microbial world and to define major phylogenetic groups. The preponderance of SSU rRNA as a phylogenetic character stemmed mostly from the fact that it is universally distributed and slowly evolving, and performs essentially the same function in all life forms. However, ribosomal RNA is not unique in this respect in that many proteins have a very wide distribution, with over 100 protein-coding genes common to all Bacteria. Analyses based on different subsets of these shared genes, ranging from single genes to extensive sequence concatenations, have resulted in trees whose topologies differ in many respects from each other and from the SSU rRNA tree. The current challenges are: 1) to recognize among the wealth of sequence information available the subset of genes, or genomic characters, that carry the least distorted phylogenetic signal and 2) to develop methods that allow for the meaningful integration of information distilled from a wide number of genes.

### The SSU rRNA Bacterial Phylogeny

The SSU rRNA tree for Bacteria currently delineates about 40 phyla, of which 13 are formed by uncultured species represented only by sequences directly cloned from the environment (Hugenholtz et al., 1998a; Hugenholtz et al., 1998b). However, the relationships among these phyla are very poorly resolved, so that, except for a few deep branching lineages, the SSU rRNA phylogeny resembles a bush rather than a tree, which is often interpreted as evidence for a rapid adaptive radiation and diversification of early bacterial life (Woese, 1987; Hugenholtz et al., 1998a; Hugenholtz et al., 1998b; Cavalier-Smith, 2002). Moreover, the addition of increasing numbers of taxa to the SSU rRNA tree has resulted in a failure to significantly support a single origin (i.e., monophyly) for some traditionally accepted phyla, such as the Proteobacteria. Indeed, although SSU rRNA clearly identifies a clade uniting the  $\gamma$ -,  $\beta$ - and  $\alpha$ -Proteobacteria (with the first two as sister groups), it does not support the inclusion of the  $\delta$ - and  $\epsilon$ -Proteobacteria (Ludwig and Klenk, 2001). The best-supported, higher-level grouping in the SSU rRNA tree unites the phyla Chlorobi (green sulfur bacteria) and Bacteroidetes (Cytophaga/Flexibacter/Bacteroides group). Rooting of the tree with archaeal and eukaryotic sequences suggests that Aquificae is the most deeply branching bacterial phylum, followed by a number of phyla represented only by environmental sequences,

and by the Thermodesulfobacteria, the Dictyoglomi and the Thermotogae. All of these deeply branching phyla are thermophilic, which, along with the fact that thermophily is thought to be ancestral in Archaea, has been taken as evidence for a “hot” origin of life (Pace, 1991). A reanalysis of SSU rRNA alignments restricted to slowly evolving positions, which are less prone to multiple substitutions and may be more reliable for early divergences (Brochier and Philippe, 2002a), groups Aquificae and Thermotogae in a derived position and places the phylum Planctomycetes as the basal lineage of the Bacteria.

### Contrasting SSU rRNA Phylogeny with Other Phylogenetic Markers

Does the topology of the SSU rRNA tree reflect a true evolutionary explosion of phyla or does this topology result from a lack of resolving power of this molecule at this deep level? These questions have been addressed by the analysis of additional genes and proteins that are shared by all bacteria. Bacterial single-gene phylogenies have been produced for a considerable number of molecules, including large subunit (LSU; 23S) rRNA (De Rijk et al., 1995; Ludwig et al., 1998; Ludwig and Klenk, 2001), elongation factor EF-Tu (Ludwig et al., 1993; Delwiche et al., 1995; Baldauf et al., 1996; Gupta, 1998a; Ludwig et al., 1998; Bocchetta et al., 2000; Jenkins and Fuerst, 2001; Ludwig and Klenk, 2001), RNA polymerase (Klenk et al., 1999; Bocchetta et al., 2000) and sigma factor (Gruber and Bryant, 1997), RecA (Eisen, 1995; Gruber et al., 1998), cytochrome (Schutz et al., 2000) and ATPase subunits (Ludwig et al., 1993; Ludwig et al., 1998; Brown and Doolittle, 1997; Ludwig and Klenk, 2001), and heat shock proteins HSP60 (Viale et al., 1994) and HSP70 (Brown and Doolittle, 1997; Gupta et al., 1997b; Gupta et al., 1998). In general, no other single phylogenetic marker provides resolution much beyond the SSU rRNA tree. Most single-gene phylogenies support the existence of the phyla delineated by SSU rRNA, and in many cases, the basal placement of the hyperthermophilic phyla. Occasionally, single-gene phylogenies support the Chlorobi-Bacteroidetes relationship seen in the rRNA tree, or indicate novel clades, sometimes in contradiction with the rRNA phylogeny. The salient features of some of these trees are summarized below.

**OTHER TRANSLATIONAL MOLECULES** The phylogeny obtained with LSU rRNA is virtually identical to that of SSU (De Rijk et al., 1995; Ludwig et al., 1998; Ludwig and Klenk, 2001), which might be expected given the functional coupling of these molecules and their potential for being

affected by the same biases on sequence evolution (G+C pressure, selection on secondary structure, etc.). Although the LSU is longer and contains more potentially informative sites than the SSU, its resolution power at the level of inter-phylum relationships is equally poor; however, Brochier et al. (2002b) have analyzed a concatenated data set containing both rRNAs and obtained some interesting higher-level groupings. In this tree, which includes only species whose genomes have been sequenced, the  $\epsilon$ -Proteobacteria are included along with the other proteobacterial classes with high bootstrap support (but the  $\delta$ -Proteobacteria were not represented in the data set). Two clades strongly supported by the rRNA concatenation are that of Cyanobacteria and *Deinococcus* and that of the hyperthermophilic phyla Aquificae and Thermotogae, represented by the genome sequences of *Aquifex* and *Thermotoga*. In addition, in the concatenated rRNA tree, the hyperthermophiles are linked with the Actinobacteria. A close affiliation between *Thermotoga* and Gram-positive bacteria (Actinobacteria and Firmicutes) has been defended by Cavalier-Smith (Cavalier-Smith, 1992; Cavalier-Smith, 2002) on ultrastructural grounds and has been suggested by phylogenetic analyses based on different proteins (Tiboni et al., 1993; Brown et al., 1994; Karlin, 1995; Gupta, 1998a).

The translation elongation factor EF-Tu is, like rRNAs, a universally distributed component of the translation machinery, and highly conservative in sequence and function owing to its basic role in the elongation of nascent polypeptides. Many phylogenetic reconstructions have employed this marker and, overall, they are in good agreement with the rRNA trees (Ludwig et al., 1993; Delwiche et al., 1995; Baldauf et al., 1996; Gupta, 1998a; Ludwig et al., 1998; Bocchetta et al., 2000; Jenkins and Fuerst, 2001; Ludwig and Klenk, 2001). Unfortunately, the resolving power of EF-Tu is inferior to that of rRNA on account of the limited numbers of informative sites, and although it supports the clustering of Chlorobi and Bacteroidetes, it does not provide any further insight into inter-phyla relationships. Even a phylogeny based on a concatenation of EF-Tu and its paralogue EF-G fails to recover the monophyly of Proteobacteria or any higher-level grouping, other than a clade uniting all phyla with the exception of the deeply branching *Deinococcus-Thermus*, Thermotogae and Aquificae (Bocchetta et al., 2000).

**INFORMATION-PROCESSING PROTEINS** Other well-conserved, information-processing molecules have been used for phylogenetic reconstruction, including RNA polymerase (Klenk et al., 1999; Bocchetta et al., 2000) and its  $\sigma^{70}$ -type sigma fac-

tor (Gruber and Bryant, 1997), and RecA (Eisen, 1995; Gruber et al., 1998). RNA polymerase (RNAP) presents the added advantage of being devoid of cases of paralogy, which often complicate protein-based phylogenies; however, the trees based on RNAP subunits are equivocal and differ from the rRNA tree in the position of the hyperthermophiles and the mycoplasmas. Instead of their commonly recovered placement at the base of the bacterial tree, Aquificae and Thermotogae branch in the proximity of Spirochaetes and Proteobacteria, while the mycoplasmas appear as the most divergent bacterial lineage. However, this result is likely an artifact resulting from the skewed base-composition and high rate of evolution of mycoplasmas because Aquificae and Thermotogae return to their usual position at the base of the tree when the mycoplasmas are removed from analysis (Bocchetta, 2000).

The phylogeny based on the  $\sigma^{70}$ -type sigma factor (a dissociable subunit of the RNA polymerase holoenzyme that confers promoter specificity) also displaces the Thermotogae from the base of the tree, grouping it with *Mycoplasma*, albeit with low support (Gruber and Bryant, 1997). In addition, this phylogeny strongly groups a monophyletic Proteobacteria (although the  $\epsilon$  class is not represented) with Chlamydiae.

RecA is a versatile protein, involved in homologous DNA recombination and in the induction of the SOS response to DNA damage. The resolving power of RecA is higher than that of other proteins in that it recognizes the monophyly of Proteobacteria, although it cannot resolve the relative branching order of the  $\delta$  and  $\epsilon$  classes. The RecA-based phylogeny corroborates very strongly the relationship between Chlorobi and Bacteroidetes, as seen in rRNA and EF-Tu trees, but it does not provide any further insight into interphylum relationships (Eisen, 1995; Gruber et al., 1998).

**PROTEINS INVOLVED IN METABOLISM AND OTHER CELLULAR PROCESSES** Numerous phylogenies have been obtained for molecules in noninformational roles, including the bioenergetics-related ATPases and cytochromes, and chaperonins. In the case of the ATPase  $\beta$ -subunit (Ludwig et al., 1993; Ludwig et al., 1998; Brown and Doolittle, 1997; Ludwig and Klenk, 2001), the resolving power of this protein is so low that monophyly is not recovered for such phyla as the Proteobacteria and the Firmicutes. On the other hand, a phylogeny based on subunits of cytochrome *bc* complexes contradicts the rRNA tree by grouping the Aquificae with the Proteobacteria (Schutz et al., 2000), as is also seen in some RNAP trees (Klenk et al., 1999; Bocchetta et al., 2000). Although the position of the Aquificae in

these trees may be due to homoplasy or horizontal transfer (Bocchetta et al., 2000; Schutz et al., 2000), other evidence hints to a relationship between the Aquificae and Proteobacteria. These two phyla share a long insertion in the RNAP  $\beta$  subunit not present in any other bacterial phylum (Klenk et al., 1999), as well as an insertion in alanyl-tRNA synthetase also seen in Chlamydiae, Chlorobi and Bacteroidetes (Gupta et al., 1999). These facts have led several authors to suggest that the Aquificae are in fact close relatives of Proteobacteria, and that their frequent position at the base of the bacterial tree is in reality an artifact caused by long-branch-attraction (Philippe and Laurent, 1998; Gupta et al., 1999; Cavalier-Smith, 2002).

The phylogenies based on the evolutionarily unrelated chaperonins HSP60 (or GroEL; Viale et al., 1994) and HSP70 (Brown and Doolittle, 1997; Gupta, 1998a; Gupta et al., 1997b) are specially interesting because they are the sole single-gene phylogenies that have been reported to divide relationships in the tight “bush”-like zone of the bacterial tree. HSP60 partitions the bacterial phyla into two well-separated clusters: 1) the Proteobacteria, Chlamydiae, Spirochaetes and Bacteroidetes, and 2) the Cyanobacteria, Actinobacteria and Firmicutes. In contrast, analyses by Gupta and coworkers (Gupta, 1998a; Gupta et al., 1997b) have found that chaperonin HSP70 includes the Cyanobacteria in cluster 1. In these phylogenies, the Deinococcus-Thermus phylum is strongly linked to Cyanobacteria, which results in a well-supported grouping of all Gram-negative phyla, and the two Gram-positive phyla (Actinobacteria and Firmicutes) are also united with relatively high support. However, archaeal genera also cluster with the Gram-positives, rendering the reliability of these HSP70-based phylogenies dubious. Moreover, a similar HSP70 analysis by Brown and Doolittle (1997) does not substantiate any significant high-level groupings among bacterial phyla.

### Post-genomic Phylogenies Based on Alignments of Extensive Data Sets

Clearly, no single molecule possesses sufficient resolving power to clarify the ancient relationships among bacterial phyla. Moreover, the conflicts between phylogenies obtained with different markers and the growing awareness of the contribution of horizontal gene transfer to the bacterial genome indicate that phylogenies based on single molecules may not be representative of the evolution of organisms and have even cast doubts on the existence of an organismal bacterial phylogeny (Gogarten, 1995; Doolittle, 1999; Zhaxybayeva and Gogarten, 2002). The recent availability of genomic

sequences for numerous bacterial species has enabled the application of very large data sets to phylogeny reconstruction. In this section, we consider the bacterial phylogenies obtained through the analysis of extensive protein sequence alignments. Two main approaches have been applied to unify the information from different molecules into a single tree: 1) concatenation of sequences for the different molecules prior to phylogenetic analysis, or 2) separate phylogenetic analysis of each molecule and a posteriori coding of the information from individual phylogenies into a single “supertree.”

**CONCATENATION-BASED ANALYSES** In the concatenation approach, each of the molecules in the analysis must be present in all species considered. Therefore, attempts to reconstruct the phylogeny of bacteria through sequence concatenations have employed different subsets of widely distributed proteins. Brown et al. (2001) utilized proteins conserved across species from all domains (Archaea, Bacteria and Eucarya), from which they removed those proteins producing trees that intermixed species from different domains (which was interpreted as evidence of horizontal transfer). The remaining subset of universally distributed proteins contained 14 information-processing molecules, mostly involved in translation. Other studies have deliberately focused on extensive concatenations of translational proteins (Brochier et al., 2002b), and more specifically, of ribosomal proteins (Hansmann and Martin, 2000; Wolf et al., 2001; M. P. Francino, unpublished analysis). Proteins of the translational apparatus, particularly ribosomal proteins, have long been thought to be less prone to horizontal transfers owing to their high level of intermolecular interactions (viz., the “complexity” hypothesis; Jain et al., 1999). Although cases of horizontal transfer of translational molecules (Doolittle and Handy, 1998; Zhaxybayeva and Gogarten, 2002), including ribosomal proteins (Brochier et al., 2000; Makarova et al., 2001; Zhaxybayeva and Gogarten, 2002) and rRNA (Yap et al., 1999), have been reported, most of these proteins seem to produce phylogenies that are congruent among themselves and with the rRNA tree, suggesting that they belong to a core set of rarely transferred genes (Brochier et al., 2002b).

The phylogenies obtained in most concatenation-based analyses retrieve the monophyly of all established bacterial phyla. In the cases where Chlorobi and Bacteroidetes are represented, they are grouped together with very high bootstrap support (Brown et al., 2001; Brochier et al., 2002b). Remarkably, these phylogenies, although not identical, recover several of the same high-level clades. For example, Chlamydiae

and Spirochaetes group together in most concatenation-based analyses (Hansmann and Martin, 2000; Brown et al., 2001; Wolf et al., 2001; Brochier et al., 2002b), a relationship not detected in single-gene phylogenies. Similarly, Aquificae and Thermotogae are often grouped together, and occur at the base in rooted versions of the bacterial tree (Hansmann and Martin, 2000; Wolf et al., 2001; Brochier et al., 2002b; M. P. Francino, unpublished analysis), although not in the analysis by Brown et al. (2001) where they remain in their usual placement as basal but separate lineages.

Most interesting are groupings that involve deeper relationships between several phyla. A cluster retrieved in several studies is that of Actinobacteria, *Deinococcus-Thermus* and Cyanobacteria (Wolf et al., 2001; Brochier et al., 2002b; M. P. Francino, unpublished analysis), although the sister relationships within the group differ among analyses. Actinobacteria and Cyanobacteria cluster together in the work of Wolf et al. (2001), whereas *Deinococcus* clusters with Actinobacteria in the work of Brochier et al. (2002b) and with Cyanobacteria in a more recent analysis with a much wider representation of cyanobacterial species (M. P. Francino, unpublished analysis). In the work of Brown et al. (2001), *Deinococcus* also groups with Cyanobacteria, but the relative placement of this cluster is not resolved. In addition, in all three analyses (Wolf et al., 2001; Brochier et al., 2002b; M. P. Francino, unpublished analysis) the Actinobacteria, *Deinococcus-Thermus* and Cyanobacteria cluster at a deeper level with the Firmicutes. Finally, another well-supported deep branch separating two large assemblages of phyla is recovered in the analyses of Brochier et al. (2002b) and Francino (unpublished analysis). This branch separates Proteobacteria, Chlamydiae, Spirochaetes, Bacteroidetes and Chlorobi (not present in Francino’s analysis) from all other phyla. As mentioned, the HSP60 tree of Viale et al. (1994) also separates Proteobacteria, Chlamydiae, Spirochaetes and Bacteroidetes from the remaining represented phyla (Actinobacteria, Cyanobacteria and Firmicutes), and no large-scale, alignment-based phylogeny has a high level of support for any branch that would contradict this grouping.

**SUPERTREE CONSTRUCTION** In an alternative approach, Daubin et al. (Daubin et al., 2001; Daubin et al., 2002) propose the independent analysis of individual protein phylogenies, followed by coding of the information obtained from each phylogeny into a matrix that will be used to generate a “supertree”. This approach is less restrictive than sequence concatenation because it can incorporate phylogenies based on different subsamples of species, and therefore



can exploit the information contained in proteins that have limited distribution. In addition, the separate phylogenetic analysis of each protein alignment enables the use of specifically optimized sequence evolution models. Daubin et al. (2002) generated trees for 310 proteins present in at least 10 bacterial species with sequenced genomes, from which they retained a subset of 120 that supported similar phylogenies and were therefore judged to represent a common history, mostly devoid of horizontal transfer. For each protein tree, supported clades were encoded in a binary matrix, with weighting proportional to the bootstrap value, and the individual matrices were concatenated into a single matrix, which was used for maximum parsimony tree reconstruction (Baum, 1992; Ragan, 1992).

The supertree obtained recovers the monophyly of all established bacterial phyla, as well as some of the high-level clades supported by other analyses. As does the analysis of translational proteins by Brochier et al. (2002b), the supertree supports the clustering of *Deinococcus* and Actinobacteria. However, unlike the majority of concatenation-based studies, the supertree does not significantly support the grouping of Cyanobacteria with *Deinococcus* and Actinobacteria, or with any other phylum. The position of these phyla in the bacterial tree is therefore still controversial. Nevertheless, several arguments favor a grouping of *Deinococcus-Thermus* with Cyanobacteria. This cluster is recovered in the large-scale studies of Brown et al. (2001) and Francino (unpublished analysis) and in the analysis of a concatenation of SSU and LSU rRNAs by Brochier et al. (2002b). Moreover, this relationship is also supported by chaperonin HSP70 trees and by patterns of indels in several proteins (see Indel Signature Sequences); Gupta, 1998a; Gupta et al., 1997b; Gupta and Johari, 1998b). The clustering of *Deinococcus* with Actinobacteria in the supertree and in the analysis of translational proteins by Brochier et al. (2002b) may be an artifact due to shared GC-rich base composition. The initial placement of the *Deinococcus-Thermus* phylum after hyperthermophiles near the base of the bacterial tree is not supported by any large-scale alignment-based studies, nor by later versions of the SSU rRNA tree (Hugenholtz et al., 1998a; b).

The supertree strongly groups the phyla Aquificae and Thermotogae, but there is no support for them as the basal lineage of Bacteria. This result mirrors that of Brochier and Philippe (2002a) on the basis of slowly evolving rRNA positions and a well-sampled representation of bacterial species. The grouping of the hyperthermophiles, although observed in many recent analyses (Hansmann and Martin, 2000; Daubin et al., 2001; Daubin et al., 2002; Wolf et al., 2001;

Brochier and Philippe, 2002a; Brochier et al., 2002b; M. P. Francino, unpublished analysis), may result from biases in nucleotide and amino acid composition due to adaptation to high temperatures. Nevertheless, the recurrence of this grouping in large-scale analyses and the relatively frequent displacement of these phyla from the base of the bacterial tree (Gruber and Bryant, 1997; Klenk et al., 1999; Bocchetta et al., 2000; Schutz et al., 2000; Daubin et al., 2001; Daubin et al., 2002; Brochier and Philippe, 2002a) seriously challenge the long held view that thermophily is the ancestral state. Instead of the hyperthermophiles, the supertree favors the Spirochaetes and Chlamydiae as the most basal bacterial lineages. Unlike large-scale concatenation-based analyses (Hansmann and Martin, 2000; Brown et al., 2001; Wolf et al., 2001; Brochier et al., 2002b), the supertree does not group these two phyla together, but rather places them as separate lineages at the base of the bacterial tree. Other phyla have also been reported to occupy the most basal position in the bacterial tree, most notably the Planctomycetes, in the analysis of slowly evolving rRNA sites by Brochier and Philippe (2002a). Hence, the resolution of the rooting of the bacterial tree still requires a much broader sample of genomic sequences for the different candidate phyla.

**INDEL SIGNATURE SEQUENCES** In addition to traditional approaches based on the analysis of nucleotide or amino acid substitutions, large sets of protein alignments have also been inspected for insertions and deletions (indels) to be employed as markers for phylogenetic reconstruction. Certain protein sequences are polymorphic for indels, and the presence or absence of particular indels can be used to assort bacteria into groups. If identical insertion or deletion events do not arise independently in different lineages, these characters could also be used to infer the branching order and relationships among organisms. Gupta (Gupta, 2000; Gupta, 2001) has shown that the occurrence of specific indels defines most bacterial phyla as cohesive groups, and by tracing the distribution of numerous indels in different proteins, he has hypothesized an order of appearance for these phyla. Although the assignment of an ancestral state is not explicit in such reconstructions, the indel “signatures” place the Gram-positive bacteria as closely aligned with the Archaea—satisfying because both have a single-membrane cell wall producing a positive Gram stain—and invoke a single origin for all two-membrane bacteria.

In conclusion, large-scale alignment-based approaches are providing new insights into deep relationships between the bacterial phyla, but different analyses are generating conflicting

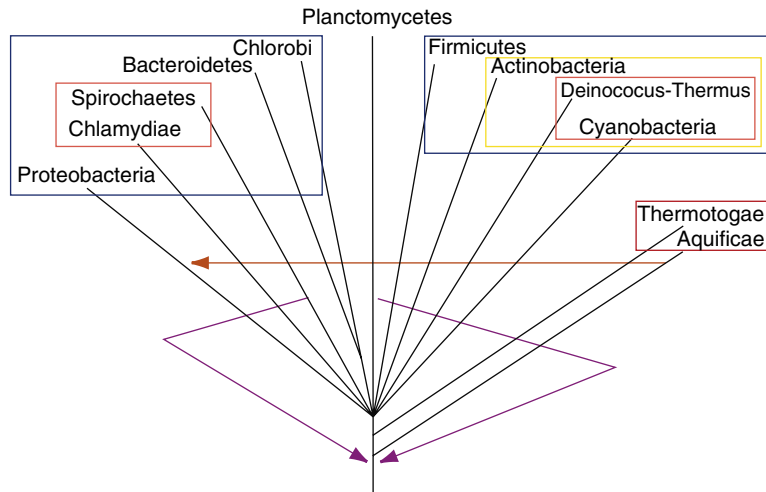


Fig. 1. Relationships among bacterial phyla most often represented in large-scale phylogenetic analyses. The cladogram depicts the basic topology of the small subunit (SSU) rRNA tree: Aquificae and Thermotogae are placed at the base, and all other phyla appear in a “bush”-like zone, where only a clade uniting Bacteroidetes and Chlorobi is resolved. Colored boxes indicate relationships strongly supported by other types of phylogenetic analyses, mostly based on protein sequence concatenations or whole-genome characters. The orange arrow indicates the proposed association between Aquificae and Proteobacteria. Purple arrows indicate alternative candidate phyla for the most basal position in the tree. The phylum Planctomycetes has no representatives for which a complete genome is available and has not been included in analyses based on extensive data sets. See text for references to the studies supporting each of the depicted relationships.

results, most notably regarding the placement of the root of the bacterial tree (Fig. 1). Although bacterial genome sequences are now appearing at a rapid rate, species sampling is still highly biased, mostly towards pathogenic and cultivable members of a restricted number of phyla. A further increase in resolution of bacterial relationships awaits a more widespread sampling of lineages. In addition, enhanced resolution will require the development of new methodologies that enable the optimal integration of information from large numbers of protein alignments. At present, the increased phylogenetic signal detected in large-scale analyses and the detection of a core of genes that have undergone similar histories (Brochier et al., 2002b; Daubin et al., 2002) suggest that there is a phylogenetic framework that reflects the evolution of substantial fractions of bacterial genomes.

## Looking Beyond Sequence Evolution

In addition to comparisons of the primary sequences of DNA or proteins, several other molecular, genetic and genomic features have been used to examine the evolutionary relationships among the Bacteria (Eisen, 2000; Wolf et al., 2002). The goal of such studies is to infer a whole-organism (as opposed to a single gene) phylogeny by developing statistics that denote

the overall similarity between entire genomes. To their advantage, these methods do not rely upon the alignments of individual genes or proteins for phylogenetic reconstruction and largely avoid inconsistencies that result from paralogy and lateral transfer unless occurring on a very broad scale. Unfortunately, the rates and patterns of evolution of many of these alternate characters are unclear such that the underlying basis of variation is not known, not specified, or not consistent among taxa. As a result, the reconstructed phylogenies are usually compared with that of SSU rRNA, and any incongruities help model the evolution of a particular trait. For example, if organisms with identical GC contents are unrelated according to their SSU rRNA sequences, the phylogenetic signal of such a character is presumed to be weak and one can conclude that the processes that modulate base composition act somewhat independently of ancestry. The problems (and arguments) arise when discordant phylogenies are inferred from characters that are each assumed to have a common basis in all organisms, to be transmitted strictly vertically, and to be resistant to convergence (such that similar traits could not have arisen independently in different taxa).

Many whole-genome characteristics can provide measures of similarity among organisms that can be used to infer phylogenetic relationships. Early genetic maps showed that both gene contents and gene order are conserved among

closely related organisms (e.g., *E. coli* and *S. typhimurium*) but that the conservation is disrupted with evolutionary distance (e.g., *E. coli* vs. *Bacillus subtilis*). Huynen and Bork (1998) compared the rates of change in gene contents and gene order with that of protein sequence evolution in 9 (8 bacterial and 1 archaeal) fully sequenced genomes. Gene order decayed most rapidly, followed by gene repertoire, and finally protein sequences remained most conserved (implying that these possess the strongest phylogenetic signal for inferring relationships among very divergent organisms). Bacterial genomes have been characterized on the basis of numerous other features, and although no explicit phylogenies are produced, these descriptors have been used to infer the intergenomic differences among organisms (Karlin et al., 2002).

### Genome Content

In a prevalent whole-genome approach, the degree of similarity between organisms is based on the fraction of genes shared. Whereas the mechanisms by which bacterial genomes can gain or lose genes are well understood, the effects of these processes and the rates at which they occur are highly variable across organisms. Because closely related organisms tend to have many uniquely shared characteristics, one anticipates that their gene contents should be similar; however, the estimated fraction of genes common to two organisms can be greatly affected by differences in genome sizes. In the worst case scenario, if a hypothetical organism contained only the minimal set of universally distributed genes, then, on the basis of its gene contents, this genome would be perceived as being equally similar to all other genomes regardless of its true ancestry. And in the reciprocal comparisons, the fraction of genes shared with this hypothetical organism would be wholly dependent upon the total gene number of a particular organism.

Despite such caveats, many of the whole genome phylogenies based on gene contents resemble those based on SSU rRNA. Naturally, the perceived similarity between genomes is influenced by how shared genes are identified. There are basically two approaches, designated by House and Fitz-Gibbon (2002) as the “homolog” method, which examines the taxonomic distribution of gene families (Fitz-Gibbon and House, 1999) or the representation of a genome in the different clusters of orthologous groups (COGs) of proteins (Tatusov et al., 1997; Lin and Gerstein, 1999; Tatusov et al., 2001; Wolf et al., 2001), and the “ortholog” method, based on the proportion of shared orthologs, usually regarded as sequences that are reciprocally the best matches in a pair of genomes (Snel et al.,

1999; Tekaia et al., 1999; Bansal and Meyer, 2002; Clarke et al., 2002; Korbel et al., 2002). The relationships between genomes by the homology method would not be affected by gene duplication or the partial loss of a gene family because these processes do not alter the absolute number of gene families, but this number is sensitive to massive genome reduction (as seen in many parasites and symbionts) and to the acquisition or loss of novel classes of genes. In contrast, the orthology method would be affected by any mechanism that removes orthologs (deletions, nonhomologous gene displacement, etc.) and by recent gene transfers and duplications.

As expected, large genomes have higher numbers of genes or gene families in common (Snel et al., 1999; Lin and Gerstein, 2000; Wolf et al., 2001; Bansal and Meyer, 2002), but when the metric is normalized by genome size, or when microbes with reduced genomes are excluded from the analysis, the distance between genomes more-or-less reflects traditional phylogenetic classifications and subdivisions. The gene-content phylogeny of Snel et al. (1999) is reminiscent of the SSU phylogeny but groups Cyanobacteria with Aquificae at the base of the bacterial tree, whereas a revised version (Huynen et al., 1999), which applies the same orthology approach but includes additional taxa, places Aquificae alone at the base and the Thermotogae with the Spirochaetes. In contrast, the genome content tree of Clarke et al. (2002), which is based on the fraction of ORFs reaching a normalized BLASTP threshold, places Thermotogae as the basal lineage. The gene content phylogenies based on the homology approach are greatly affected by the inclusion of organisms with highly reduced genomes (which tend to group together), and more “phylogenetically reasonable clades” (Wolf et al., 2001) are recovered by omitting these taxa. Looking at the shared occurrence of gene families in the genomes of sequenced, free-living organisms, House and Fitz-Gibbon (2002) place the Aquificae at the base of the bacterial tree, but the branching order of other bacterial phyla remains obscure.

### Ortholog Similarity

Although different pairs of orthologs conserved between two genomes can display dissimilar levels of identity as a result of differential functional constraints, the distribution of distances between orthologs should reflect the evolutionary relationship between a given pair of genomes. Several authors (Grishin et al., 2000; Clarke et al., 2002; Wolf et al., 2002) have identified orthologs common to two fully sequenced genomes by searching for the reciprocal best hits and used various measures to convert ortholog similarity

scores to a matrix conveying the pairwise distance between genomes. Depending on which taxa are included in the particular study, the phylogenies extracted from these distance matrices differ in placing Thermotogae, Aquificae or both at the base of the bacterial tree.

### Gene Order

Early comparisons of sequenced genomes detected little long-range conservation in gene order (Mushegan and Koonin, 1996), but overlaid on this pattern, there are several gene clusters and gene pairs (mostly involving ribosomal proteins) maintained in all bacterial genomes (Siefert et al., 1997; Dandekar et al., 1998; Wachtershauser, 1998; Wolf et al., 2001). Conservation of gene order seems to reflect the evolutionary distance between organisms (Tamames, 2000; Wolf et al., 2001), but it evolves faster than gene contents (Huynen and Bork, 1998; Korbelt et al., 2002), and its use as a phylogenetic marker should be limited to comparisons of rather closely related genomes (Suyama and Bork, 2001). Kunisawa (2001) has developed a method for reconstructing phylogenetic relationships based on the physical arrangement of clustered genes, and when applied to members of the Proteobacteria, this method produces a topology that is consistent with the traditional sequence-based classifications.

### Other Genome Features

Because of the well-known heterogeneity in the sizes and nucleotide compositions of bacterial genomes, there have been attempts to group bacterial taxa according to total G+C contents, codon usage biases, di- tri- and tetra- nucleotide frequencies (genomic signature abundances), the number and distribution of repetitive sequences, short palindromes and rRNA operons, gene and protein lengths, and amino acid composition (reviewed in Karlin et al., 2002). These approaches have retrieved some intriguing (coincidental?) similarities between genomes that are not typically grouped in sequence-alignment-based phylogenies, but few of these features can be viewed as a consistent and reliable indicator of evolutionary distance because many are susceptible to convergence.

Wolf et al. (1999) and Lin and Gerstein (2000) have taken a unique approach by examining the occurrence of specific protein folds throughout the genome, and the phylogenetic groupings in the resulting “fold” trees are surprisingly similar to those based on SSU rRNA. Several authors have begun to make direct comparisons of the tree topologies of Bacteria recovered from different features of the genome and proteome (Lin

and Gerstein, 2000; Wolf et al., 2001; Wolf et al., 2002). These studies clearly show that some aspects of the genome contribute only weak phylogenetic signals and imply that the addition of characters to an analysis, even those that are known to be genetically determined, will not always increase the accuracy of the tree.

## Enterobacteriaceae: What’s in a Name?

Even for the most extensively studied group of the Bacteria, recent molecular phylogenetic analyses have revealed some unrecognized kinships between taxa as well as numerous inconsistencies in the present classification scheme. The Enterobacteriaceae are Gram-negative, facultatively anaerobic rods belonging to the  $\gamma$ -class of Proteobacteria. Only a few actually reside in an “enteric” environment; however, most fall within a group of related species that colonize the mammalian intestine. There are currently 41 described genera in *Bergey’s Manual of Systematic Bacteriology* 2<sup>nd</sup> edition (Garrity, 2001), including species found in terrestrial and aquatic environments, and associated with plants or animal hosts. Members of this family are usually motile, catalase positive, oxidase negative, and use the Embden-Meyerhof pathway to catabolize sugars and to produce acids from the fermentation of glucose. They can be distinguished from other Gram-negative, rod-shaped bacteria (such as those in the families Vibrionaceae and Pasteurellaceae) by cell geometry, flagellar arrangement, oxidase production, sodium requirements and the presence of a common antigen (Brenner, 1981).

Despite these and other shared metabolic properties, assignment of microorganisms to the Enterobacteriaceae no longer requires detailed information about the morphological and biochemical characteristics of an organism. Molecular phylogenetic analyses have now defined the extent of sequence variation within this taxonomic group, and any strain whose sequence falls within this delineated range can be tentatively classified in the Enterobacteriaceae, even without knowledge of its morphology, metabolic capabilities, or growth requirements. Of course, this assumes that the phylogenetic trees and evolutionary relationships based on genes represent those of the organisms themselves, and at least for many of the species constituting the Enterobacteriaceae, the correspondence between the molecular and the original phenotypic classification schemes is rather good. That said, we should note that molecular analyses have revealed that several enteric genera are polyphyletic, such that their constituent species are not derived from a

unique common ancestor. For example, some species of *Klebsiella* and *Enterobacter* are more closely related to one another than to other species typed to the same genus (Harada and Ishikawa, 1997; Mollet et al., 1997; Hauben et al., 1998; Dauga, 2002). Such findings have resulted in the recent reclassification of certain taxa and changes in nomenclature. At present, the determination of a comprehensive phylogeny encompassing all genera within a family cannot yet rely on whole-genome sequencing, and relationships at this and lower taxonomic levels are still determined through single-gene phylogenies, mostly SSU rRNA.

### Phylogenetic Analyses of Enterobacteriaceae Based on SSU rRNA

The phylogenetic tree in Fig. 2 displays the evolutionary relationships of many well-recognized genera of Enterobacteriaceae and, for reference, *Haemophilus influenzae* and *Vibrio cholerae*, which are nonenteric members of the  $\gamma$ -Proteobacteria. This phylogeny is derived from SSU rRNA sequences; and in many cases, relation-

ships among the better-studied organisms match those resolved by biotyping. It is evident that certain genera are cohesive and monophyletic, that is, contained within a group that consists of a single common ancestor and all of its descendant organisms. As mentioned above and as previously reported (Hauben et al., 1998; Sproer et al., 1999; Dauga, 2002), *Klebsiella*, *Enterobacter* and *Serratia* are polyphyletic, consisting of species descended from two or more ancestors.

To construct the phylogeny in Fig. 2, SSU rRNA sequences for representatives of all recognized enteric genera (except *Moellerella*, *Saccharobacter*, *Trabulsiella* and *Yokenella* for which cognate sequences are not available) were extracted from GenBank. For several genera, numerous sequences are available (either from multiple species within the genus or from multiple rRNA operons within a genome) yielding a set of 193 enteric SSU rRNA sequences, which were aligned with ClustalX using the default settings (Thompson et al., 1997). Following initial alignment, sequences were removed if they were not full length or if the genera and/or species were not well represented in the literature. Next,



Fig. 2. Phylogeny of Enterobacteriaceae based on small subunit (SSU) rRNA. See text for criteria used to select species and for methods of phylogenetic reconstruction.

Table 1. Sample information for Enterobacteriaceae sequences used in the phylogenetic analyses.

Bacterial species	Strain	Accession no.
<i>Brenneria (Edwinia) salicis</i>	DSM 30166	AJ233419
<i>Buchnera aphidicola</i>	Sg ( <i>Schizaphis graminum</i> )	NC_004061 <sup>a</sup>
<i>Citrobacter freundii</i>	DSM 30039	AJ233408
<i>Edwardsiella tarda</i>	NB8031	AB050832
<i>Enterobacter cloacae</i>	CCCO10	AF511434
<i>Enterobacter intermedius</i>	ATCC 33110	AF310217
<i>Erwinia amylovora</i>	BC205	AF141896
<i>Escherichia coli</i>	O157 : H7	NC_002695 <sup>a</sup>
<i>Haemophilus influenzae</i>	KW20	NC_000907 <sup>a</sup>
<i>Hafnia alvei</i>	ATCC 13337	M59155
<i>Klebsiella planticola</i>	ATCC 33531T	AF129443
<i>Klebsiella pneumoniae</i>	AU1496	AY043391
<i>Morganella morganii</i>	M567	AF461011
<i>Pantoea stewartii</i>	DC283	AJ311838
<i>Pectobacterium (Erwinia) chrysanthemi</i>	582	AF373175
<i>Photorhabdus asymbiotica</i>	ATCC 43950	Z76755
<i>Plesiomonas shigelloides</i>	ATCC 7966T	X60418
<i>Proteus vulgaris</i>	ATCC 29906T	AJ301683
<i>Providencia stuartii</i>	ATCC 29914T	AF008581
<i>Rahnella aquatilis</i>	CDC 1327-79	U90757
<i>Salmonella bongori</i>	BR1859	AF029227
<i>Salmonella enterica</i> sv. Typhi	CT18	NC_003198 <sup>a</sup>
<i>Serratia grimesii</i>	LMG 7883	AF286868
<i>Serratia marcescens</i>	AU1388	AY043389
<i>Shigella dysenteriae</i>	ATCC 13313	X96966
<i>Vibrio cholerae</i>	ATCC 14033	X74694
<i>Xenorhabdus nematophila</i>	CB6	AF522294
<i>Yersinia enterocolitica</i>	ATCC 9610	AF366378
<i>Yersinia pestis</i>	CO92	NC_003143 <sup>a</sup>

<sup>a</sup>Genbank accession number of completely sequenced genome.

all but one (or two) sequences were removed from the set for each genus if the multiple representatives for a given genus were found to be monophyletic. The resulting data set contained 27 taxa, including 22 enteric genera (Table 1). The NEXUS file containing these sequences is available at the TreeBase Web site (<http://www.treebase.org>) under Study Accession S801 and Matrix Accession M1267.

To refine the sequence data for phylogenetic analysis, two short regions (positions 70–103 and 461–489) were excluded owing to high levels of sequence and length variation, which made alignments ambiguous. To determine the best-fit model of DNA evolution, the resulting alignments were subjected to hierarchical likelihood ratio tests in Modeltest v3.06 (Posada and Crandall, 1998). For the phylogenetic analyses, a neighbor-joining (NJ) tree was constructed under the general time reversible (GTR) model of evolution (log likelihood = -6952.9580; estimated by Modeltest v3.06) with Phylogenetic Analysis Using Parsimony (PAUP\* v4.0b10; Swofford, 2000). The maximum parsimony (MP) analysis was conducted using PAUP\* by optimizing characters with accelerated transformation, 10 repetitions of random sequence additions,

starting trees obtained by stepwise addition, and branches swapped by tree bisection-reconstruction. In addition, a Bayesian inference of phylogeny was conducted using MrBayes v2.01 (Huelsenbeck and Ronquist, 2001b). The GTR model, with some sites assumed to be invariant and with variable sites assumed to follow a discrete gamma distribution, was used for all analyses. Bayesian analyses were initiated with random starting trees and run for 1.5 million generations, with a 100 generations sampling interval. Replicates were performed to determine that analyses were not trapped at local optima (Huelsenbeck and Bollback, 2001a). After discarding the initial burn-in samples (the first 5,000 trees), the remaining 10,000 trees were used to generate a 50% majority rule consensus tree using PAUP\*, with the percentage of trees containing a node representing that node's posterior probability (Huelsenbeck and Ronquist, 2001b).

Figure 2 represents the most reliable composite estimation of the phylogenetic relationships among enteric bacteria. A time scale for the diversification of bacterial species has been proposed (Ochman and Wilson, 1987; Doolittle et al., 1996) on the basis of the estimated divergence time of 100 million years ago for the split

between *E. coli* and *Salmonella enterica*. If SSU rRNA evolves at approximately the same rate in all enteric taxa (Ochman et al., 1999), then it is possible to estimate the divergence time between any pair of taxa by extrapolation.

Structural RNAs evolve very slowly and are not useful for resolving the relationships among closely related organisms, such as strains within a species or, sometimes, even species within a genus. In addition to SSU rRNA, several other molecules and typing methods have been used to examine relationships among enterics, and below, we briefly discuss the phylogenetic status of species within representative enteric genera:

*Escherichia/Shigella* The genus *Escherichia* contains five species: *Escherichia blattae*, *E. coli*, *E. fergusonii*, *E. hermannii* and *E. vulneris* whose relationships to one another, and to other enteric species, have been resolved by the phylogenetic analysis of *gapA* and *ompA* sequences (Lawrence et al., 1991). *Escherichia coli* and *E. fergusonii* form a monophyletic clade, with each being more closely related to one another than either is to *Salmonella*; however, the other three species classified in this genus represent a phylogenetically heterogeneous group of organisms. *Escherichia hermannii* is basal to *E. coli*, *Salmonella enterica* and *Citrobacter freundii*; *E. blattae* branches between *Serratia* and *Klebsiella*; and *E. vulneris* is polyphyletic, with one strain grouping with *K. pneumoniae*.

The relationships of thousands of strains of *E. coli* have been determined by multilocus enzyme electrophoresis, and several other typing schemes have examined the genetic variation in clinical, laboratory and natural isolates (Whittam, 1997). It is probably safe to say that more is known about the degree, appointment and maintenance of genetic variation in *E. coli* than in any other bacterial species. The genera *Escherichia* and *Shigella* were grouped together by DNA-DNA hybridization studies, which demonstrated that they belonged to the same genetic species (Brenner et al., 1972; Brenner et al., 1973). Subsequent analyses have shown that the variation within the four recognized species of *Shigella* (*S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*) is contained within that observed in *E. coli*, i.e., strains of *Shigella* have arisen through multiple independent origins from within *E. coli* (Ochman et al., 1983; Pupo et al., 1997; Pupo et al., 2002).

*Salmonella* The genus *Salmonella* is monophyletic and comprises two species, *Salmonella bongori* and *S. enterica*. The separation of *S. bongori* from *S. enterica* has been confirmed by DNA-DNA hybridization (Le Minor et al., 1982), phylogenetic analyses of housekeeping and rRNA

genes (Selander et al., 1996; Christensen et al., 1998b). *Salmonella bongori* is considered to be a homogenous group (Hoszowski and Wasyl, 2000), whereas *S. enterica* is subdivided into seven subspecies, enumerated as I, II, IIIa, IIIb, IV, VI and VII, which encompass more than 2,000 serovars. Most human pathogens (e.g., Typhi) belong to subspecies I. The relationships within *Salmonella* have been derived from multilocus enzyme electrophoresis and by the analysis of several genes, which have been sequenced in each of the subspecies type strains constituting the *Salmonella* Reference Collection (SARC; Boyd et al., 1996; Brown et al., 2002).

*Erwinia/Pectobacterium/Brenneria/Pantoea* Originally, the genus *Erwinia* was divided into two taxonomic groups, *amylovora* and *cartovora*, on the basis of phytopathogenicity properties (Brown et al., 2000). The *amylovora* group is considered less metabolically active and requires organic nitrogen for growth, whereas the *cartovora* group has higher metabolic activity, reduces nitrates to nitrites, and causes soft-rot diseases (Brenner, 1981). Subsequently, phylogenetic analyses of SSU rRNA demonstrated that the genus *Erwinia* was polyphyletic, which led to the partitioning of *Erwinia* into three genera: *Erwinia*, *Pectobacterium* and *Brenneria* (Hauben et al., 1998). The genera *Erwinia* and *Brenneria* contain the necrogenic phytopathogens (formerly the *amylovora* group) and *Pectobacterium* encompasses the soft-rotting phytopathogens (formerly the *cartovora*). An additional group of phytopathogens closely related to *Erwinia* has been assigned to the genus *Pantoea*.

*Serratia* Members of the genus *Serratia* can be found in environmental samples, as well as in association with human disease. There are nine described species within the genus: *Serratia odorifera*, *S. marcescens*, *S. rubidaea*, *S. entomophila*, *S. ficaria*, *S. fonticola*, *S. plymuthica*, *S. proteamaculans* and *S. grimesii* (Sproer et al., 1999). Phylogenetic analyses of SSU rRNA sequences suggest that *Serratia* is polyphyletic, with one cluster more closely related to *Hafnia alvei* and *Obesumbacterium proteus*, than to the other *Serratia* cluster (Sproer et al., 1999). In contrast, phylogenetic analyses of the sequences encoding the ATPase domain of DNA gyrase (*gyrB*) showed *Serratia* to contain two clusters forming a monophyletic group (Dauga, 2002).

*Klebsiella* Klebsiellae are detected in environmental samples as well as on the mucosal surfaces of mammals. Certain species are opportunistic pathogens and are associated with respiratory diseases and urinary tract infections in humans (Podschun and Ullman, 1998). The

five most common species are *Klebsiella aerogenes*, *K. edwardsii*, *K. pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis*, and in some classifications, *K. edwardsii* and *K. pneumoniae* are subdivided into additional subspecies (Podschun and Ullman, 1998). Phylogenetic analyses of SSU rRNA (Hauben et al., 1998), translation initiation factor 2 (*infB*; Hedegaard et al., 1999), RNA polymerase  $\beta$ -subunit (*rpoB*; Mollet et al., 1997), *groE* (Harada and Ishikawa, 1997) and *gyrB* (Dauga, 2002) all suggest that the genus is polyphyletic. In these studies, some species of *Klebsiella* were more closely related to members of the genus *Enterobacter* than to other *Klebsiella* species (Hauben et al., 1998; Dauga, 2002). In light of this evidence, there is a need to reclassify the members of this genus in a way that reflects their molecular relationships.

*Enterobacter* The *Enterobacter* occupy similar environmental and ecological niches as *Klebsiella*. There are 16 described species within the genus, and as with *Klebsiella*, the genus *Enterobacter* is polyphyletic. On the basis of the phylogenetic analyses of nucleic acid and protein-coding sequences, certain *Enterobacter* species should be grouped with *Klebsiella*. For example, *E. aerogenes* falls in a clade with *Klebsiella pneumoniae* rather than with *E. cloacae* in phylogenies based on SSU rRNA and *gyrB* sequences (Dauga, 2002).

OTHER ENTERIC GENERA Some of the other members of the Enterobacteriaceae exhibit a wide range of associations with eukaryotic hosts, ranging from opportunistic pathogens to mutualistic symbionts. Some examples of pathogens include *Edwardsiella* (which are pathogenic to fish), certain *Yersinia* and *Citrobacter* (which cause diarrhea and disease in humans), and *Proteus* (which induce otitis in dogs). In contrast, *Photorhabdus* and *Xenorhabdus* consist almost entirely of mutualistic symbionts of the nematode genera *Steinernema* and *Heterorhabditis* (Boemare et al., 1993). These symbionts serve to maintain conditions conducive for nematode reproduction (Poinar and Thomas, 1966; Poinar and Thomas, 1967) and produce antimicrobial compounds that inhibit the growth of other microorganisms (Akhurst, 1983; Boemare et al., 1993).

The phylogenetic relationships within several of these enteric genera have been analyzed using SSU rRNA sequences (e.g., *Buttiauxella* [Sproer et al., 1999], *Photorhabdus* and *Xenorhabdus* [Suzuki et al., 1996; Liu et al. 1997], and *Yersinia* [Ibrahim et al., 1993]). And for those enteric genera containing but a single species (e.g., *Calymmatobacterium* [Kharsany et al., 1999], *Candidatus Phlomobacter fragariae* [Zreik et al.,

1998], and *Hafnia* [Hauben et al., 1998; Sproer et al., 1999]), relationships to other members of the Enterobacteriaceae (as well as identification as unique genera) have typically been established by the analysis of SSU rRNA sequences.

## The Emergence of Bacterial Symbionts

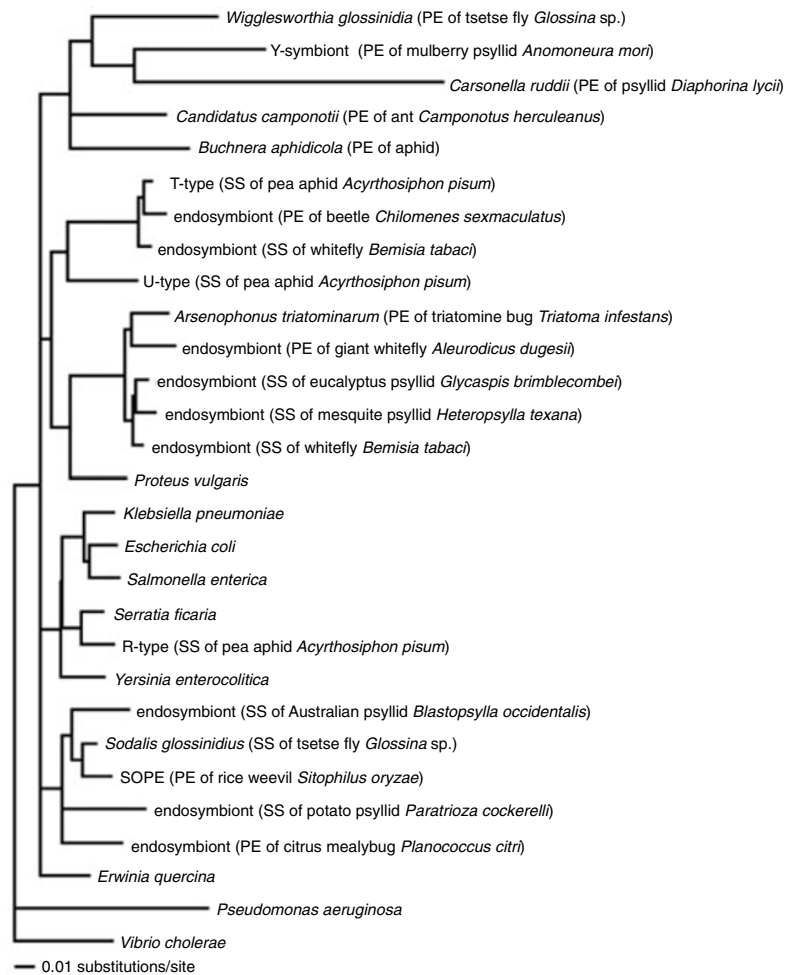
The characterization of bacterial symbionts, which are broadly distributed and cannot be maintained outside of their animal hosts, has been one of the greatest successes of culture-independent methods for the identification and classification of microbes. Insects that feed exclusively on blood or plant sap contain obligate (primary) symbionts that offset their host nutritional deficiencies, reside within specialized host organs, and are essential for host survival. In addition to these primary symbionts, many insects contain secondary symbionts that confer no obvious benefit to their host, are not essential for either host growth or reproduction, and invade a diverse range of host tissues. Whereas primary symbionts are strictly vertically transmitted within a host lineage, sometimes for hundreds of millions of years (as evident from the concordance between symbiont and host phylogenies), secondary symbionts can be horizontally transmitted among insect host species (Moran and Baumann, 2000).

Molecular phylogenetic analyses have provided evidence that the majority of primary and secondary symbionts of insects have evolved from within the Proteobacteria, and in particular the  $\gamma$ -Proteobacteria (Fig. 3). To show the relationships of symbiotic lineages to the Enterobacteriaceae, we included several enteric species in this phylogeny and rooted this tree with *Vibrio cholerae*, which also serves as an outgroup to the enteric bacteria (Fig. 2).

The evolution of symbionts from free-living proteobacterial ancestors often involves a vast reduction in genome size; in fact, at 440 kb, the bacterial symbiont of aphids has the smallest known genome for a cellular life form (Gil et al., 2002). As is apparent from this phylogeny, the origin of new symbiotic species can occur by two routes: 1) the independent derivation of symbionts from free-living ancestors, and 2) the diversification from within a clade that is already symbiotic (usually associated with the infection of a new host species). The molecular genetic information for most of the symbionts included in Fig. 3 is presently limited to SSU rRNA sequences; however, their small genome sizes and limited gene repertoires make many of these bacteria ideal subjects for genome projects.



Fig. 3. Small subunit (SSU) rRNA phylogeny of  $\gamma$ -proteobacterial symbionts and related free-living species. Many symbionts have no official nomenclature and are listed according to their designations in the literature or in sequence databases. Common names and Latin binomials of hosts are given. PE, primary endosymbiont; SS, secondary symbiont.



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## The Genus *Escherichia*

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### Introduction

*Escherichia coli*, originally called “*Bacterium coli* commune,” was first isolated from the feces of a child in 1885 by the Austrian pediatrician Theodor Escherich (Escherich, 1885). *Escherichia coli* is a common inhabitant of the gastrointestinal tract of humans and animals. There are *E. coli* strains that are harmless commensals of the intestinal tract and others that are major pathogens of humans and animals. The pathogenic *E. coli* are divided into those strains causing disease inside the intestinal tract and others capable of infection at extra-intestinal sites (Kaper et al., 2004). *Escherichia coli* is easily cultured in the clinical laboratory, but the identification of the different pathogenic genotypes requires virulence gene detection methods not typically available in most clinical laboratories. *Escherichia coli* can be found secondarily in soil and water as the result of fecal contamination. Classically, its detection has been used as an indicator of poor water quality. From biochemical, physiological and genetic perspectives, *E. coli* is one of the best understood and characterized living organisms, with laboratory studies on model strains such as *E. coli* K-12 taking place over the past sixty years (extensively reviewed in *Escherichia coli* and *Salmonella: Cellular and Molecular Biology*, third edition; available online through the <http://www.asmpress.org/browse/virtual/index>.

asp?SelectedItem=10075{ASM Press Web site}). Shown in Table 1 are additional websites providing general and specific *E. coli* portals for useful genetic and metabolic information.

### Taxonomy and Phylogeny

#### General Comments

The comparative analysis of 5S and 16S ribosomal RNA sequences suggest that *Escherichia* and *Salmonella* diverged from a common ancestor between 120 and 160 million years ago, which coincides with the origin of mammals (Ochman and Wilson, 1987). *Escherichia* and *Shigella* have been historically separated into different genera within the Enterobacteriaceae. DNA sequence analysis of their genomes reveals a high degree of sequence similarity and suggests to many bacteriologists that they should be considered a single species (see *Shigella* genome references: Ewing et al., 1958; Kimura, 1980; Brenner, 1984; Saitou and Nei, 1987; Simmons and Romanowska, 1987; Nei and Miller, 1990; Pupo et al., 2000; Jin et al., 2002; Wei et al., 2003). Currently, the two organisms continue to be discussed as two different genera anchored in the historical perception of their disease potential and ecology. Besides *E. coli*, there are other species within the genus, *E. adecarboxylata*, *E. blattae*, *E. fergusonii*, *E. hermannii* and *E. vuln-*

Table 1. *E. coli* reference sources on the Internet.

Name of Web site	Web site Address	Information Provided
International <i>E. coli</i> Alliance <i>E. coli</i> Database Portal	<a href="http://www.uni-giessen.de/~gx1052/IECA/ieca.html">http://www.uni-giessen.de/~gx1052/IECA/ieca.html</a>	General portal site for <i>E. coli</i> information
The <i>E. coli</i> Index	<a href="http://ecoli.bham.ac.uk/">http://ecoli.bham.ac.uk/</a>	General portal site for <i>E. coli</i> information
Center for Disease Control: Foodborne and Diarrheal Diseases Branch	<a href="http://www.cdc.gov/ncidod/dbmd/foodborne/index.htm">http://www.cdc.gov/ncidod/dbmd/foodborne/index.htm</a>	CDC informational site for food-borne pathogens including <i>E. coli</i>
CGSC: <i>E. coli</i> Genetic Stock Center	<a href="http://cgsc.biology.yale.edu/">http://cgsc.biology.yale.edu/</a>	Provides database of <i>E. coli</i> genetic information includes genotypes and reference information for the strains in the CGSC collection, gene names, properties, and linkage map, gene product information and information on specific mutations

*eris*. Little is known about the distribution, biology or interrelatedness of these species.

### *Escherichia coli* Clonality

Evolutionary studies based on either DNA sequence analysis or multilocus enzyme electrophoresis has identified clonal phylogenetic groupings of *E. coli*. Phylogenetic studies have principally used the *E. coli* reference (ECOR) strain collection as a common reference for current evolutionary comparisons (Ochman and Selander, 1984). This collection can be acquired from Thomas Whittham's laboratory (<http://foodsafety.msu.edu/whittam/ecor/>). Six phylogenetic groups are generally recognized among the ECOR strains (A, B1, B2, C, D and E; Selander et al., 1987; Fig. 1). For the phylogenetic groups, there are some general biotype clusterings (e.g., raffinose fermentation is common among B1, C, D and E strains, whereas sorbose fermentation is common to B2 strains; Miller and Dancel, 1986). There are limited instances where host sources are associated with clonal types. For example, among the original B1 strains, many were commonly isolated from herbivores (Selander et al., 1987). In general, however, there is notably little association between host strain source and clonal designation.

### Evolution of Pathogenic Types (Pathotypes) of *E. coli*

Virulent strains of *E. coli* are differentiated clinically from one another on the basis of epidemiology, signs and symptoms of their respective diseases, microscopic observations of their interactions with host cells, and of biotypes and unique gene markers. The specifics for each of the *E. coli* pathotypes will be discussed in the disease topic below. The evolution of independent pathogenic types of *E. coli* is striking and to date unmatched by any other bacterial genus. How this occurred is unclear, but it is likely linked to the concomitant evolution of different mammalian hosts. Initially isolates involved in both intestinal and extra-intestinal human disease were thought to be concentrated mostly within the single, B2 ECOR phylogenetic group with a smattering of isolates found in the D group. However, recent studies indicate that extensive horizontal transmission of blocks of genes has occurred across the different phylogenetic clonal lines. Thus, with the possible exception of the enterohemorrhagic *E. coli* O157:H7, many of the *E. coli* pathotypes apparently do not have unique evolutionary origins. The virulent *E. coli* strains have arisen independently on multiple occasions within clonal lines (Pupo et al., 1997). On the basis of the relative number of

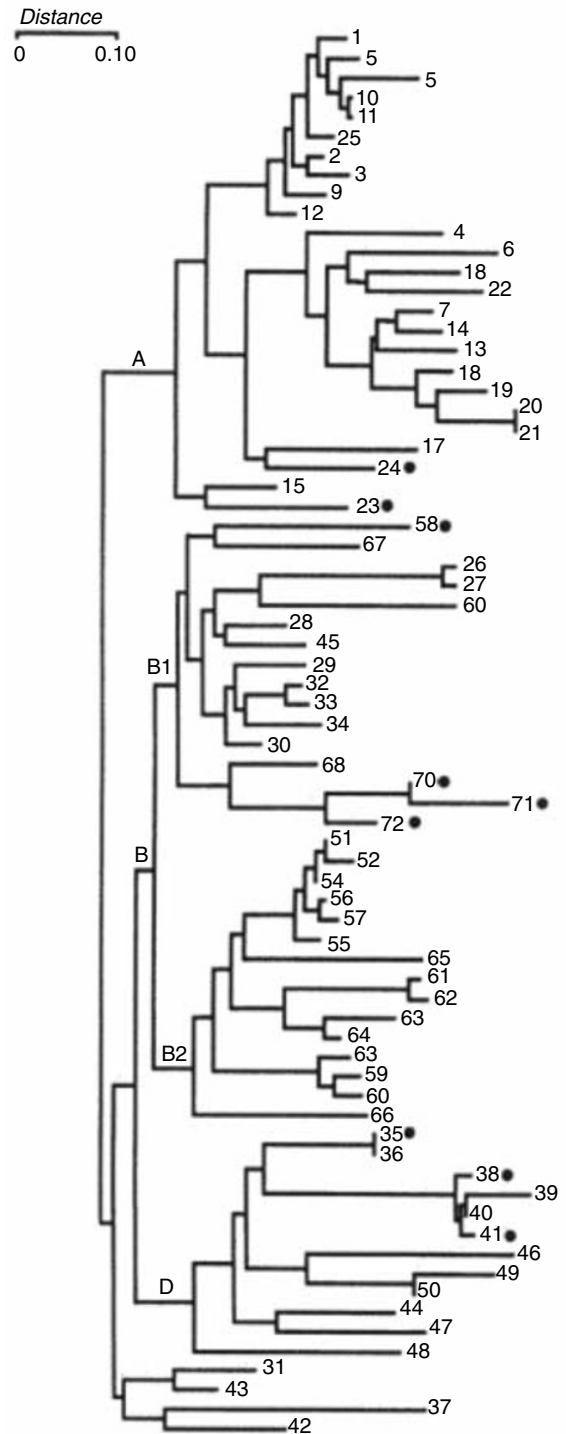


Fig. 1. Phylogenetic distribution of multicopy single-stranded DNA (msDNA)-containing *E. coli* strains of the *E. coli* reference (ECOR) collection. The tree was inferred by the neighbor-joining (NJ) algorithm applied to the genetic distances based on polymorphisms of 38 enzyme-encoding loci. Circles represent the nine ECOR strains in which msDNA was detected by the RT-labeling method.

isolates identified within the general diarrheagenic and extra-intestinal pathotype groupings, the former are more frequently found in the A, B1 and D phylogenetic groups, whereas the extra-intestinal *E. coli* strains are more common to the B2 lineage (Johnson, 2002).

### Evolution of the *E. coli* Genome

Genome sequencing of three different *E. coli* strains (laboratory K-12 strain MG1655, enterohemorrhagic O157:H7 strain EDL933, and an uropathogenic isolate, CFT073) reveals an unambiguous conservation of nearly 40% of the core gene sequences among the three isolates (Welch et al., 2002). The synteny of the genes around the circular chromosomes is nearly intact and representative of the classic *E. coli* K-12 gene map (Berlyn, 1998). The phylogenies used to build the ECOR phylogeny is also reflected in the relatively slow divergence of the core gene sequences. The general conservation of genes among the three isolates undoubtedly reflects the physiological nature of what can be termed “*E. coliness*.” *Escherichia coli*, even the pathogenic types, at some point thrive in a mammalian intestine, yet they are also capable of surviving periods in the outside environment. However, there is striking evidence that concurrent with the vertical evolutionary processes that account for the ECOR-based phylogenetic differences, horizontal genetic transfer has occurred frequently and had the greatest impact on genetic differences among strains. Comparisons of one genome with another reveal hundreds of instances where insertions, substitutions and deletions of large blocks of DNA have disrupted the order of the core genes. For example, the CFT073 genome is nearly 600 kbs larger than MG1655, and in one-on-one comparisons, there are approximately 1100 genes unique to K-12 and greater than 1800 genes unique to CFT073 (Fig. 2). The majority of the unique genes are on segments that vary from 4 kb to over 100 kb. Aside from complete and partial prophages, the largest blocks of strain-specific genes share some common features. They are often located at tRNA genes (e.g., *leuX*, *selC*, *argW*, *pheV* and *pheU*). The G+C content is often lower than the typical 50–52 mol% for the core genes. The predicted codons used in the EDL933 or CFT073 strain-specific genes is skewed towards greater use of tRNAs typically less abundant than those involved in *E. coli* K-12 translation. These observations suggest that the larger gene blocks originate in and are mobilized from genera much different than any of the close relatives of *E. coli* (such as *Salmonella*). Prior to genomic sequencing, it was recognized that many of the most significant virulence genes (adhesins, extracellu-

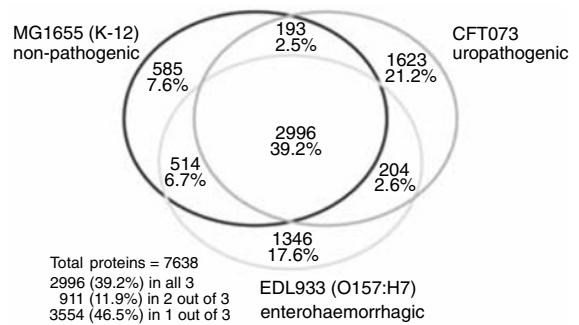


Fig. 2. Shared *E. coli* proteins. Comparison of the predicted proteins of the three *E. coli* strains shows the number of orthologs in each shared category and numbers of strain-specific proteins. Hypervariable proteins and proteins extending across junctions between island and backbone sequences were excluded from the analysis. Number of proteins counted: K-12 (4288), CFT073 (5016), and EDL933 (5063). In the totals for the three strains, orthologous proteins are counted only once. Orthologous proteins meet the same match criteria used for designation of the backbone.

lar protein secretion systems, and toxins) for several *E. coli* pathotypes were clustered together in these large blocks. Hacker and colleagues coined the term “pathogenicity-associated islands” (PAIs) to describe these Hacker et al., 1997). Still unclear is whether much of the added, unique genetic material has anything to do with pathogenicity. Thus, the unique gene clusters are often simply and appropriately called “genetic islands.” The ability to build a phylogeny for some of the *E. coli* pathotypes is complicated because the horizontally acquired islands do not always share chromosomal location and genetic content (Welch et al., 2002).

### Habitat

*Escherichia coli* are common inhabitants of the terminal small intestine and large intestine of mammals. They are often the most abundant facultative anaerobes in this environment. They can occasionally be isolated in association with the intestinal tract of nonmammalian animals and insects. The presence of *E. coli* in the environment is usually considered to reflect fecal contamination and not the ability to replicate freely outside the intestine. There is evidence however to suggest that *E. coli* may freely replicate in tropical fresh water (Bermudez and Hazen, 1988).

### Cell Structure

*Escherichia coli* are Gram-negative, nonspore-forming bacilli. They are approximately 0.5  $\mu\text{m}$  in diameter and 1.0–3.0  $\mu\text{m}$  in length. Within the periplasm is a single layer of peptidoglycan. The



peptidoglycan has a typical subunit structure where the *N*-acetylmuramic acid is linked by an amide bond to a peptide consisting of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid and finally D-alanine.

*Escherichia coli* are commonly motile in liquid by means of peritrichous flagella. Swarming behavior and differentiation into hyperflagellated and elongated bacilli typical of that seen with the *Proteus* species can be observed on some solid media (Harshey, 1994). *Escherichia coli* are commonly fimbriated. The type 1 pili are the most common and are expressed in a phase switch ON or OFF manner that leads to piliated and nonpiliated states (Eisenstein, 1987). One of the traits commonly encoded on the larger genetic islands of the different pathotypes of *E. coli* are additional pili (chaperone-usher and type IV pili families and non-pili adhesins; Bann, 2002; Schreiber and Donnenberg, 2002).

Among *E. coli* isolates, there is considerable variation and many combinations of somatic (O and K) and flagellar (H) antigens. Among pathogenic strains, there are few patterns of these antigens and few phylogenetic groupings (see below). For *E. coli*, there are over 150 antigenically unique O-antigens (Whitfield and Valvano, 1993). K type capsular material occurs in two or four forms on the basis of physical, biochemical and genetic criteria (Jann and Jann, 1990; Whitfield and Roberts, 1999). Over 80 serologically and chemically distinct capsular polysaccharides have been reorganized (Jann and Jann, 1992). In addition, a slime layer, colonic acid extracellular polysaccharide, is common to many *E. coli* isolates and can be co-expressed with some K-type capsules (Keenleyside et al., 1993). There are 53 H-antigen specificities among *E. coli* (Wang et al., 2003).

## Cell Physiology

*Escherichia coli* is a facultative anaerobe. It is capable of reducing nitrates to nitrites. When growing fermentatively on glucose or other carbohydrates, it produces acid and gas. By traditional clinical laboratory biochemical tests, *E. coli* is positive for indole production and the methyl red test. Most strains are oxidase, citrate, urease and hydrogen sulfide negative. The classic differential test to primarily separate *E. coli* from *Shigella* and *Salmonella* is the ability of *E. coli* to ferment lactose, which the latter two genera fail to do. Aside from lactose, most *E. coli* strains can also ferment D-mannitol, D-sorbitol, and L-arabinose, maltose, D-xylose, trehalose and D-mannose. There are limited instances where pathogenic strains differ from the commensals in their metabolic abilities. For example, commensal *E. coli* strains generally use sorbitol, but *E.*

*coli* O157:H7 does not. Most diarrheagenic strains cannot utilize D-serine as a carbon and nitrogen source, but uropathogenic and commensal fecal strains can use this enantiomer of serine (Roesch et al., 2003).

Most *E. coli* strains are capable of growing over a wide range in temperature (approximately 15–48°C). The growth rate is maximal in the narrow range of 37–42°C (Ingraham and Marr, 1987). *Escherichia coli* can grow within a pH range of approximately 5.5–8.0 with best growth occurring at neutrality. Some diarrheagenic *E. coli* strains have the ability to tolerate exposure to pH 2.0. Such an acid shock mimics transit through the stomach and induces expression of sets of genes involved in survival and pathogenesis (Waterman and Small, 1996).

There are several sources of information on genes identified as essential for growth of laboratory strain K-12. Efforts are underway to identify essential genes for drug target studies, as well as to construct a freely replicating *E. coli* strain with the smallest possible genome. The study of such an engineered strain would simplify the analyses of regulatory circuits and mechanics of fundamental processes such as cell division and chromosomal replication (Gerdes et al., 2003); information on the topic with links can be found at the (<http://www.genome.wisc.edu/resources/essential.htm>{University of Wisconsin Essential genes in *E. coli* Web site}). information about profiling the *Escherichia coli* chromosome can be found at the (<http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp>{Shigen *E. coli* Web site}).

Iron metabolism of *E. coli* is an especially well-studied topic (Braun and Braun, 2002). Ferric iron is brought into *E. coli* by chelating compounds such as citrate, enterobactin, aerobactin, yersinabactin and heme. These chelators each have highly specific outer membrane proteins that enable their uptake across the outer membrane where they are then brought across the cytoplasmic membrane by ATP binding cassette (ABC) transport systems. One trait that sets many of the pathogenic *E. coli* apart from the normal intestinal *E. coli* is the ability to acquire ferric iron from a wide array of chelators. The multiple gene systems enable adaptation to sites where iron might be limited by host antibacterial activities (Torres et al., 2001). These virulence-enhancing iron acquisition systems, such as aerobactin, are often encoded by plasmids or are present on pathogenicity islands.

## Genetics

A great deal of what is known about bacterial genetics comes from the study of the *E. coli* laboratory strain K-12. K-12 was isolated in 1922

from the stool of a convalescent diphtheria patient (Lederberg, 1951). Although K-12 strains are rough in terms of their O-antigen, physical and genetic analyses indicate that the original K-12 parent was likely to have been an O16 strain Liu and Reeves, 1994). In general, wild strains of *E. coli* are capable of gene transfer by conjugation and transduction, albeit at rates that are greatly reduced when compared to K-12 laboratory events. Natural competency for DNA transformation has not been observed for *E. coli*, although genes similar in sequence to *Hemophilus influenzae* competence genes are present in the *E. coli* K-12 genome Finkel and Kolter, 2001). In the laboratory, artificial methods such as electroporation enable the uptake of DNA and its stable maintenance through recombination or replication as a plasmid or prophage. Conjugal, as well as nonconjugal, plasmids are commonly found in wild strains of *E. coli*.

## Ecology

*Escherichia coli* strains are commonly carried in the mammalian intestine. Usually a predominant clone can be found at any given time, but other less abundant clones can be found simultaneously. Whittham's lab demonstrated that in a study of healthy 3–6 year old girls the most abundant *E. coli* clones varied when examined on a weekly basis. In some rare instances a single clone could be found over a 4-week period, and in others an individual may have as many as 16 different clones over the same period. The average was three clones per girl over the 4-week period. Therefore, the *E. coli* strains found in the intestine are multiclonal and fluctuate in their predominance over time. Colonization of the periurethral area or urinary tract occurs briefly, and the strains isolated at these sites are usually not the predominant clone in the stool at the same time (Schlager et al., 2002).

Aside from adhesins and metabolic traits that would favor one *E. coli* strain over another in their ability to colonize particular sites, the production and immunity to bacteriocins undoubtedly affects the dynamics of the persistence and dominance of individual strains (Riley and Gordon, 1999). Bacteriocin production may give a special competitive advantage in nutrient-poor environments Riley and Gordon, 1999).

## Disease

### *Escherichia coli* Pathotypes

One of the most notable features of *E. coli* is broad diversity of disease-causing genotypes. As

mentioned above, the diseases can encompass different symptoms and gastrointestinal tract pathologies, but there are also diseases at extraintestinal sites. These different genotypes and their disease-causing abilities lead to categories of *E. coli* often referred to as pathotypes. There are six intestinal and two extraintestinal pathotypes currently recognized Nataro and Kaper, 1998a; Nataro et al., 1998b; Schreiber and Donnenberg, 2002; Kaper et al., 2004). Enterotoxigenic *E. coli*.

A frequent cause of diarrhea in both humans and animals, enterotoxigenic *E. coli* (ETEC) are estimated to cause 600 million cases of human diarrhea and 800,000 deaths worldwide principally in children under the age of 5 (World Health Organization, 1999). Economically significant ETEC diarrheal disease in animals occurs in neonatal calves, pigs and lambs. ETEC cause watery diarrhea that can be mild in nature or in some instances can be a severe, cholera-like illness where rapid dehydration can be life-threatening. In endemic areas of ETEC-mediated diarrhea, infants and children under the age of 5 are the most commonly affected. ETEC exposure in endemic areas is one of the most common causes of traveler's diarrhea.

One of the principal virulence factors for this pathogen is the heat-labile enterotoxin (LT), which interestingly shares structural and functional similarity to the *Vibrio cholerae* cholera toxin (Sixma et al., 1991; Sixma et al., 1993; Spangler, 1992). LT has a classic AB toxin subunit holotoxin structure. The B subunits (as a pentamer) bind to host cell surface GM1 and GD1b gangliosides and the A subunit enzymatically ADP-ribosylates the  $\alpha$ -subunit of stimulatory G protein. This G protein regulates host cell adenylate cyclase and LT-mediated modification leads to its permanent activation and an increase in intracellular cAMP levels. This eventually leads to activation of the chloride ion channel of the intoxicated cells, increased chloride ion secretion into the intestinal lumen, and decreased sodium and chloride absorption. The overall result is to reverse the normal intestinal osmotic gradient and cause a net water loss into the gut lumen. Aside from LT, many ETEC strains also express heat-stable enterotoxins (STs), which also contribute to the watery diarrhea. There are two structurally distinct STs, STa and STb. The STs are small polypeptides that share the common features of heat stability and multiple intramolecular disulfide bonds. The action of STa is well understood. It binds to the extracellular domain of plasma membrane-embedded guanylate cyclase. The ETEC toxins are secreted in the terminal small intestine where the ETEC adhere by expression of a complex and diverse group of surface proteins commonly

referred to as “colonization factors” (Gaastra and Svennerholm, 1996).

### Enteropathogenic *E. coli*

These organisms are a significant cause of infant diarrhea in developing nations. Enteropathogenic *E. coli* (EPEC) were historically recognized on the basis of serotypes such as O55:H6 and O127:H6. They are currently defined as those diarrheagenic *E. coli* strains that cause attaching and effacing (A/E) lesions on intestinal epithelium but which lack Shiga toxins (verotoxins). There is a great diversity of the *E. coli* serotypes that possess these features. This makes the serotype classification scheme ineffective and indicates that there may be a diversity of pathogenic mechanisms and evolutionary lineages. EPEC disease is generally the result of growth of EPEC in the small intestine. EPEC cause a watery diarrhea that may contain mucus but typically does not have blood in it. Vomiting, fever, malaise and dehydration are also associated. The symptoms may last for a brief period of several days, although instances of long, chronic EPEC disease have been noted.

Some of the mechanisms of EPEC pathogenesis are well understood. For example, the A/E lesion is the result of a complex system of EPEC proteins that are injected into the host intestinal epithelial cell. The A/E lesion represents a dramatic rearrangement of the epithelial cytoskeleton where there is an accumulation of actin directly below the attached EPEC cell. This is often described as an actin pedestal for the attached bacterial cell. There is a specific pathogenicity island, termed the “locus of enterocyte effacement” (LEE), that encodes the genes responsible for the A/E lesion (McDaniel et al., 1995). The LEE encodes a type III secretion system that provides the intimate adhesin, its receptor (which is injected into and then presented on the surface of the host cell), and the injected proteins responsible for changes in host cell signaling mechanisms (including actin pedestal formation; Jerse, 1990; Kenny et al., 1997). Common to most EPEC strains are plasmids, termed “EAF” (“EPEC adherence factor”) plasmids, which encode an adherence factor, the bundle-forming pilus (bfp; Nataro et al., 1987; Donnenberg and Kaper, 1992; Sohel et al., 1996). Results of human volunteer studies indicate the EAF plasmid is necessary to cause disease (Levine et al., 1985). Although the A/E characteristic is critical for causing EPEC disease, probably through destruction of microvilli, the precise mechanism for the diarrhea is not completely understood and may reflect the diversity of EPEC strains. For example, some but not all EPEC produce an enterotoxin, EspC (Mellies, 2001).

Recent attention has focused on greater understanding of atypical EPEC strains (Trabulsi et al., 2002). These strains more commonly cause diarrhea in industrialized nations than the typical EPEC strains. In addition the atypical EPEC strains have animal and human reservoirs, whereas the typical isolates are almost always associated with human fecal contamination. The atypical isolates have the ability to cause A/E lesions but lack the EAF plasmids. They often have additional virulence factors not seen among the typical strains. For example, they have significant portions of the pO157 virulence plasmid common to enterohemorrhagic *E. coli* O157:H7 strains and may have a heat stable enterotoxin (EAST-1).

### Enterohemorrhagic *E. coli*

These organisms share the ability to cause A/E lesions with EPEC but enterohemorrhagic *E. coli* (EHEC) are set apart from EPEC by possession of Shiga-like toxins and the clinical presentation of their disease. EHEC cause disease of the large intestine that may present as simple watery diarrhea and then progress to bloody stools with ulcerations of the bowel. In a small subset of diseased individuals there is onset several days later of severe, life-threatening hemolytic-uremic syndrome (HUS). HUS involves a triad of hemolytic anemia, thrombocytopenia and renal failure. The transmission of EHEC disease in humans is through ingestion of contaminated beef or foods contaminated with cattle feces. In cattle, the EHEC strains are transient members of the intestinal microflora where they do not apparently cause disease. One of the remarkable features of EHEC is its low infection dose of 10–100 organisms. Clearly this microorganism has special acid-tolerance ability when compared to many other enteric bacterial pathogens. Children under the age of five are the major victims of EHEC disease, although the elderly may also exhibit bloody diarrhea and HUS. Epidemiologically in the United States, Japan, and Great Britain, a single serotype O157:H7 is the most common EHEC strain. In other parts of the world, this strain can be observed causing disease, but other serotypes (e.g., O26 and O111) cause a similar disease as well.

All factors that lead to HUS are unknown except Shiga toxin (sometimes referred to as “Shiga-like toxin” or “verotoxin”), which probably plays an important role in renal injury. Purified Stx-1 injected intravenously in baboons leads to renal disease with histopathology similar to EHEC-mediated HUS (Taylor et al., 1999). The Shiga toxin inhibits protein synthesis through cleavage of ribosomal RNA. Because EHEC do not cause bacteremia, Shiga toxin is thought to

be released while the organism is growing in the large bowel, where it gets disseminated systemically to cause damage to renal endothelial cells and release of inflammatory mediators that eventually damage the kidney. There are two evolutionarily related forms of Shiga toxin in *E. coli* (Shiga toxin 1 and Shiga toxin 2). They share approximately 55% amino acid sequence similarity. Shiga toxin 1 is only different from the Shiga toxin of *Shigella dysenteriae* by a single amino acid substitution.

There are many Shiga toxin positive *E. coli* strains (STEC) that are not associated with enterohemorrhagic colitis. It is a heterogeneous group that is occasionally associated with HUS, but their general benign nature may be due to their lack of the LEE pathogenicity island and plasmid virulence factors. The ubiquitous dissemination of the distribution of Shiga toxin genes among *E. coli* strains is due to their transmission as part of lambdoid phages. The EHEC O157:H7 strain likely originated in an O55 EPEC strain where a series of genetic events lead to acquisition of shiga toxin-encoding prophages and a large virulence plasmid, pO157 (Reid et al., 2000; Lathem et al., 2003). The precise role of pO157 in EHEC pathogenesis is unknown but may involve some putative toxin genes and a mucin-specific zinc metalloprotease, StcE (Burland et al., 1998; Lathem et al., 2002; Gryns et al., 2005).

#### Enteroaggregative *E. coli*

These organisms are defined as *E. coli* that do not possess LT enterotoxin or Shiga toxins but adhere to cultured HEp-2 cells in self-aggregates that are classically referred to as “stacked bricks” (Nataro et al., 1987). Clearly, many *E. coli* strains can mediate the “stacked brick” adhesive phenotype, but there is a subset of these that are bona fide human diarrheal pathogens. Enteroaggregative *E. coli* (EAEC) disease, as described by human volunteers, is a watery diarrhea that occurs in some cases with abdominal cramps, but no fever (Nataro et al., 1995). There is no invasion of the bloodstream. The disease seen in natural EAEC outbreaks is often reported as a persistent, seemingly chronic watery diarrhea. These small epidemics occur in both developing as well as industrialized countries. There are no common serotypes of EAEC to aid in their recognition in the clinical laboratory. The pathogenesis of EAEC disease is poorly understood, although several potential virulence factors are common to EAEC isolates. EAEC express a fimbrial adhesin called “aggregative adherence fimbriae” (“AAF”). EAEC isolates often produce a mucinase called “Pic” whose gene has the ability to express from its nonencoding DNA strand a

smaller gene that encodes an enterotoxin (*Shigella* enterotoxin [ShET1]) first described in *Shigella* strains. EAEC strains often produce a heat stable enterotoxin EAST1 that is homologous to the ST1 of ETEC.

#### Diffusely Adherent *E. coli*

The epidemiology and pathogenesis of the diffusely adherent *E. coli* (DAEC) are not well understood. DAEC may cause diarrhea in very young children (less than a year old; Scaletsky et al., 2002). They are differentiated from the other diarrhegenic *E. coli* by a distinct adhesion phenotype, again on HEp-2 cells. The adhesion is brought about by F1845 fimbriae, which belong to the Dr family of adhesins (also found in some UPEC strains). The Dr adhesins recognize and bind to host cell surface decay accelerating factor (DAF). DAEC bound to cultured cells elicit a cytopathic phenotype and activation of signal-transduction pathways. The relative significance of DAEC as a pathogen and its mechanisms for causing disease await further study.

#### Enteroinvasive *E. coli*

These organisms are pathogenetically so closely related to *Shigella* species that the nomenclature distinction is questionable. There are a few biochemical traits that can be used to distinguish enteroinvasive *E. coli* (EIEC) from *Shigella*, but the principal virulence genes are shared. The diagnostic confusion between *Shigella* and EIEC is evident in that EIEC isolates are nonmotile and 70% are nonlactose fermenters (Silva et al., 1980). In addition, EIEC share with *Shigella* the inability to decarboxylate lysine, a trait common to other *E. coli*. The traits that EIEC share with *E. coli* but not *Shigella* are the ability to produce gas from glucose and fermentation of xylose.

EIEC cause invasive inflammatory colitis and dysentery with a clinical presentation (blood and mucous stools accompanied by fever and severe cramps) identical to the disease caused by *Shigella* species. EIEC/*Shigella* invade intestinal epithelium, principally in the large intestine. Once inside the cells, they lyse the phagocytic vesicle and replicate freely in the host cell cytoplasm. The EIEC/*Shigella* cells then spread to neighboring host cells by a motility process whereby actin is nucleated on one pole of the bacillus and subsequent actin polymerization propels the bacterial cell (Goldberg and Theriot, 1995). Many of genes necessary for cellular invasion and disease are carried on a large >200-kb plasmid found in both EIEC and *Shigella*. A system of type III secretion genes important for delivery of modifiers of host cell signaling and membrane lysis are found on these plasmids. In

addition, the plasmid encodes an outer membrane protein (IcsA) that is localized on one pole of the bacterium and directs the actin microfilament polymerization necessary for spread of bacteria to other host cells. EIEC/*Shigella* rarely invade the bloodstream, but they do invade the lamina propria immediately under the intestinal epithelium, where interaction with macrophages causes the release of pro-inflammatory mediators and even induction of apoptosis. Interestingly, the inability to decarboxylate lysine, a trait shared by EIEC and *Shigella*, is the result of mutations and gene rearrangements at the *cadC* gene. The decarboxylation of lysine results in cadaverine, which acts as an inhibitor of inflammation and migration of neutrophils into the lamina propria. The lack of this function is hypothesized to be a pathoadaptive trait that enables EIEC/*Shigella* to cause disease (Maurilli et al., 1998; Fernandez et al., 2001; Casalino et al., 2003).

**EXTRAIESTINAL *E. COLI*** Two separate pathotypes of *E. coli* are generally recognized causes of extraintestinal human diseases (neonatal septicemia/meningitis *E. coli* [meningitis-associated *E. coli*, MAEC] and the urinary tract and bloodstream *E. coli* [uropathogenic *E. coli*, UPEC]). Some isolates, *E. coli* O18:K1:H7, are recognized as having the potential to cause both invasive neonatal diseases and urinary tract infections (UTI; Johnson et al., 2001a; Johnson et al., 2001b).

UPEC are a heterogeneous group of clones (Donnenberg and Welch, 1996). Within the UPEC grouping are cystitis, pyelonephritis and urosepsis isolates. These strains are the principal causes of morbidity and mortality from either community or hospital-acquired *E. coli* infections. Approximately 60% of adult women will have a UTI in their lifetimes (Kunin, 1994). As much as 90% of all community-acquired UTIs and greater than 30% of the hospital-acquired UTIs are caused by *E. coli* (Haley et al., 1985). There have been reports of community-wide outbreaks of UTIs by multidrug resistant UPEC clones (Manges et al., 2001).

UPEC strains isolated from women with pyelonephritis, but who have no underlying medical complications, often possess specific O serotypes (O1, O2, O4, O6, O7, O18 and O75; Orskov and Orskov, 1983a; Orskov and Orskov, 1983b; Orskov and Orskov, 1985; Johnson et al., 1987; Wold et al., 1992). What further suggests that these *E. coli* strains are extraordinary is that they are especially capable of invading the bloodstream (Johnson et al., 1987; Johnson et al., 1988; Johnson, 1991a; Johnson et al., 1991b; Johnson et al., 1994). Many of the known or putative virulence factors for these strains are not shared with

common fecal *E. coli* strains. Examples of such factors are adhesins (e.g., Pap, Sfa, and Dra), hemolysin (Hly), cytotoxic necrotizing factor-1 (CNF-1), and the aerobactin (Aer) iron-sequestration systems (reviewed by Donnenberg and Welch, 1996). Recently, a member of the autotransporter protein family, Sat, has been demonstrated to be a cytotoxin of uroepithelial cells (Guyer et al., 2000). There are additional factors that are common to all *E. coli* that are critical for pathogenesis of extraintestinal disease. The principal factors are lipopolysaccharide, capsule production, and type 1 pili. The type 1 pili appear to play a particularly critical role in the initial colonization of the bladder (Kisielius et al., 1989; Connell et al., 1996; Langermann et al., 1997; Lim et al., 1998; Struve and Krogfelt, 1999). The type 1 pili have recently been shown to mediate cellular invasion of cultured urinary epithelial cells (Mulvey et al., 1998). It is suggested that intracellular cellular invasion leads to persistent infections of the urinary tract by successive rounds of intracellular infection, multiplication, release and reinfection of superficial, as well as deeper bladder epithelial layers (Mulvey et al., 2001). Currently no information is available about genes other than those for type 1 pili that are needed for cellular invasion.

The first described pathogenicity islands were found to encode the non-type 1 adhesins (e.g., pap, sfa, and dra), toxins (e.g., Hly, CNF1 and Sat), and iron-sequestration systems (e.g., aerobactin; Knapp et al., 1986; Hacker et al., 1990; Blum et al., 1994; Blum et al., 1995). The chromosomal endpoints of these UPEC islands are associated with specific tRNA loci *leuX*, *selC*, *pheV* and *pheU*. Often there are phage integrase-like genes present on one end of the islands. This has led to a common perception that nonpathogenic organisms become pathogens by the acquisition of these virulence genes en bloc via prophages that use the tRNA genes as attachment sites. Comparison of genome sequences of three different *E. coli* strains indicates in excess of 300 unique loci, which appear as insertions or substitutions along the length of these genomes. Clearly there is a subset 10–13 tRNA gene loci where genetic differences among the strains occur uniformly (e.g., *selC*, *pheV* and *leuX*). Therefore, phages probably participated in their acquisition and evolution. There are, however, many more sites where the genomes differ and it is unclear what genetic mechanisms lead to these differences.

**MENINGITIS-ASSOCIATED *E. COLI*** Along with Group B streptococci, meningitis-associated *E. coli* (MAEC) are the most common causes of neonatal meningitis, a severe disease with a high mortality rate and possible long-term neurologi-

cal problems in survivors (Unhanand et al., 1993; Stoll et al., 2002). There are a limited number of *E. coli* serotypes associated with this disease, but greater than 80% of the strains express K1 capsule (Robbins et al., 1974; Sarff et al., 1975). It is generally thought that the newborn acquires the K1 strain from its mother during passage through the birth canal. The strain then progressively invades the bloodstream and subsequently crosses endothelial surfaces into the brain. The K1 capsule is a critical determinant in invasion across the blood-brain barrier. In a rat pup model, the capsule was proven to be necessary for survival in the meninges (Hoffman et al., 1999). S-fimbriae enable K1 isolates to adhere to brain microvascular endothelium (Parkkinen et al., 1988). Several genes, *ibeA*, *ibeB*, *ibeC*, *cnf-1* and *aslA*, are required for endothelial cell invasion (Prasadarao et al., 1999; Badger et al., 2000a; Khan et al., 2002). Also common to most MAEC isolates is a 100-kb plasmid that confers increased virulence (Mercer et al., 1984; Badger et al., 2000b). The best-characterized MAEC isolate is RS218, which possesses that classic O18:K1:H7 serotype. Its genome sequence has been determined, although not presented in a published form as of the end of 2004 (see the <http://www.genome.wisc.edu/sequencing/rs218.htm>{*E. coli* genome project}). Preliminary studies indicate that like the other *E. coli* pathotypes, the K-12, EHEC or UPEC genomes do not contain a significant number of genes unique to RS218. In a genomic comparison of RS218 to K-12, at least 500 kb of DNA are unique to RS218 (Rode et al., 1999; Bonacorsi et al., 2000). See Table 1 for *E. coli* reference sources on the Internet.

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## The Genus *Edwardsiella*

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### Taxonomy

In 1965, Ewing and colleagues proposed the creation of a new species, *Edwardsiella tarda*, to house a collection of 37 strains that were primarily of fecal origin. These strains, extensively studied at the United States Centers for Disease Control and Prevention (CDC) since 1959 and referred to as “bacterium 1483–59,” were sufficiently distinct biochemically from other taxa within the family Enterobacteriaceae to warrant this proposal. Simultaneously, a Japanese group (Sakazaki et al., 1965) had been independently studying a collection of 256 Japanese cultures, mostly from snakes, with similar, though not identical, biochemical properties to bacterium 1483–59 (Farmer and McWhorter, 1984). These reptilian isolates were given the vernacular name “Asakusa Group,” a reference to the place from which they were originally isolated. Others, most notably King and Adler (1964) and Rakovskí and Aldov (1965), described the “Bartholomew Group,” which was a collection of yet other unidentified Enterobacteriaceae strains isolated from diarrheal stools with properties similar to *E. tarda*. This group of organisms took its name from the county hospital in Columbus, Indiana, where the first fecal isolate had been recovered.

Don Brenner at the Walter Reed Army Institute of Research, in a DNA hybridization study (Brenner et al., 1974), tested the validity of *E. tarda* as a new taxon within the Enterobacteriaceae and its relationship to the other phenotypically similar groups (Asakusa, Bartholomew). Polynucleotide sequence data indicated 1) the relatedness of *E. tarda* to *Escherichia coli* K-12 was only 20%, and 2) all 20 strains of *E. tarda*, isolated from diverse sources and geographic locales, formed a single highly related (88% at 60°C) hybridization group. Furthermore, by DNA-DNA hybridization, members of the bacterium 1483–59, and the groups Asakusa and Bartholomew were found to belong to the same species, *E. tarda* (Table 1). These studies conclusively established the legitimacy of the genus and species, *Edwardsiella tarda*, and the relationship

between this taxon and the Asakusa and Bartholomew groups.

In 1975, Sakazaki and Tamura challenged the species name “*tarda*” over the epithet “*anguillimortiferum*” based upon an earlier published species designation, *Paracolobactrum anguillimortiferum*, proposed in 1962 by Hoshina, for an organism recovered from eels. This bacterium had biochemical properties identical to those of *E. tarda* in tests performed in common. However, Hoshina (1962) failed to designate a type strain, and the original culture of *P. anguillimortiferum* was lost in the intervening years. Because “*anguillimortiferum*” was validly published first and had taxonomic priority over “*tarda*,” Sakazaki and Tamura (1975) proposed the name *E. anguillimortifera* (Hoshina) comb. nov. to replace the illegitimate combination *E. tarda*. Invoking the first principle of the *International Code of Nomenclature of Bacteria*, which calls for preserving nomenclatural stability and avoiding confusion, Farmer et al. (1976) subsequently argued for keeping the more widely used *tarda* over *anguillimortiferum*. Although an opinion was never formally rendered, the situation was resolved by the publication of the approved list of bacterial names in January, 1980 (Skerman et al., 1980). Although both *E. tarda* and *E. anguillimortifera* (p. 292) appeared on the approved list of bacterial names as objective synonyms, *E. tarda* was designated the type species. Ultimately, Sakazaki and Tamura (1991) recommended that the name *E. tarda* be retained based upon its common usage in most scientific reports.

The genus *Edwardsiella*, named in 1965 after P.R. Edwards (a CDC microbiologist) to honor his numerous contributions to the field of enteric bacteriology, contained only a single species until 1980 (Sakazaki and Tamura, 1991). Then in rapid succession two new species were added. First, Grimont et al. (1980) published data on a group of malonate- and D-mannitol-positive strains recovered from reptiles and birds, which, based upon DNA-DNA reassociation kinetics, constituted a new species. The name *E. hoshinae* was proposed for this new group, in honor of the

Table 1. Reassociation kinetics of *E. tarda* strains to *E. tarda* (CDC 3592-64).

CDC strain no.	Descriptor	Relative binding (%)		Divergence (%)
		60°C	75°C	
3592-64	Asakusa group, Japan	100	100	NT
5497-61	Asakusa group, Japan	85	84	0.1
1483-59	<i>E. tarda</i> type strain, Kentucky	91	NT	0.1
1795-62	Original Bartholomew strain	84	82	0.3

CDC, US Centers for Disease Control and Prevention; NT = not tested.

Data from Brenner et al. (1974).

Japanese bacteriologist Toshikazu Hoshina; CIP 78-56 (ATCC 33379) was designated the type strain. To date, the report by Grimont et al. (1980) is the only publication in the literature on this *Edwardsiella* species. Hawke et al. (1981) described a third *Edwardsiella* species, *E. ictaluri*, from isolates of channel (*Ictalurus punctatus*) and white (*Ictalurus catus*) catfish. Five of 13 *E. ictaluri* were 81–97% related to the type strain ATCC 33202 (CDC 1976–78) in DNA-DNA reassociation studies, whereas *E. tarda* and other members of the Enterobacteriaceae were only 56–61% and 6–41% related to *E. ictaluri*, respectively. Results of multilocus enzyme (isozyme) electrophoresis conducted on 33 isolates for 23 loci indicate *E. ictaluri* is an extremely homogeneous species with a mean genetic diversity of 0.033 and an average of 1.304 electromorphs per loci (Starliper et al., 1988). Currently then, the genus *Edwardsiella* has three species with taxonomic standing. The type species for the genus is *E. tarda* (type strain ATCC 15947) and the G+C content of *Edwardsiella* species is 53–59 mol% (Sakazaki and Tamura, 1991).

## Phylogeny

The genus *Edwardsiella* is a member of the Enterobacteriaceae based upon biochemical and physiologic characteristics (Brenner, 1984), the presence of the enterobacterial common antigen (Mikeli and Mayer, 1976), and DNA relatedness, albeit distant, to core genera in this family (including *Escherichia*; Brenner, 1977). Thus by definition, *Edwardsiella* is a member of subgroup 3 of the  $\gamma$  subclass of the class *Proteobacteria* (superfamily I), formerly known as purple bacteria (Woese et al., 1985; Stackebrandt et al., 1988; Logan, 1994).

It is somewhat difficult to find the correct phylogenetic position of *Edwardsiella* within the family Enterobacteriaceae, which has been overclassified for medical purposes and has roughly the equivalent genetic diversity of a single genus such as *Bacillus* (Ahmad et al., 1990; Logan, 1994). This means that phylogenetic trees constructed from 16S rRNA sequence analysis and

DNA-rRNA hybridization data are not always useful in determining the correct evolutionary picture at the genus and species level (Logan, 1994). In addition, 16S rRNA and signature sequences for *Edwardsiella* and *E. tarda* have not been formally published (Stackebrandt et al., 1988) and many evolutionary trees do not contain *Edwardsiella* (MacDonell et al., 1986; Ahmad et al., 1990; Logan, 1994).

At the DNA level, the genus *Edwardsiella* is only ~20% related to *E. coli* and to other core genera of the family Enterobacteriaceae including *Salmonella*, *Shigella* and *Citrobacter* (Brenner, 1977). In investigations conducted by Brenner et al. (1974), the highest degree of DNA relatedness between *E. tarda* CDC 3592-64 and other genera in this family was noted with *Hafnia* (29%) and *Serratia* (28%). The large evolutionary distance between *Edwardsiella* and these core genera is further supported by molecular analysis of housekeeping genes.

The phylogenetic branching of biochemical pathways can be used to indicate points of divergence among various prokaryotic microorganisms; this is particularly true for amino acid biosynthesis. For instance, for genes regulating aromatic amino acid biosynthesis (e.g., of tryptophan), all enterobacteria have a T protein (chorismate mutase-cyclohexadienyl dehydrogenase) and three regulatory isozymes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. However, they differ in respect to the presence of an anthranilate synthetase: anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase gene fusion (*trpG-trpD*), the presence or absence of cyclohexadienyl dehydratase and of a chorismate mutase species free of any known association (Ahmad and Jensen, 1988; Ahmad et al., 1990). A phylogenetic tree based upon 16S rRNA data and biochemical character states (i.e., aromatic amino acids) identifies three enteroclusters, the third of which contains the genera *Edwardsiella*, *Cedecea*, *Kluyvera*, *Hafnia*, *Yersinia*, *Proteus*, *Providencia* and *Morganella* (Ahmad et al., 1990). In *E. coli*, the leader regions of the *ilvGMEDA* operon, which controls expression of branched-chain amino acids (isoleucine, leucine and valine), is similar to the

*ilvG* region of other enteric bacteria (Harms et al., 1985; Williamson and Jackson, 1987). The *ilv* leader region sequence of *Salmonella* and *Klebsiella* and that of *E. coli* have >96% homology (Harms et al., 1985). In contrast, the sequence diversity between either *Edwardsiella tarda* or *Serratia marcescens* and *E. coli* is 25–29%. The tRNA<sup>Leu</sup> sequence of *E. coli* is homologous with the leader peptide-coding region of the *ilvG* leader transcript of *E. coli*, *S. typhimurium* and *Klebsiella aerogenes* but not with that of *Serratia marcescens* or *E. tarda* (Williamson and Jackson, 1987).

Results reported in the analysis of amino acid biosynthesis genes are similar to those reported in other housekeeping genes. TonB homologues are involved in the transport of ferric siderophores and are present in most enterobacteria excluding *Morganella*. However, unlike most core enterobacteria (*E. coli*, *Shigella* and *Citrobacter*), *Edwardsiella tarda* only reacts weakly with  $\alpha$ -TonB polyclonal antiserum and not at all with several TonB-specific monoclonal antibodies (Larsen et al., 1996). In yet another example, cloned genes from two *E. coli* catalases (*katG*, *katE*) used as probes in Southern hybridization assays failed to detect analogous sequences in other Enterobacteriaceae including *E. tarda*, *Proteus mirabilis*, *S. marcescens* and *Erwinia carotovora* isolates (Switala et al., 1990). Cumulatively, these results suggest a distant phylogenetic relationship between edwardsiellae and core members of the Enterobacteriaceae such as *E. coli*.

No formal 16S rRNA (small-subunit) phylogenetic trees of the family Enterobacteriaceae that include the genus *Edwardsiella* have been published (Stackebrandt et al., 1988); the same is true for 5S rRNA cluster analysis (MacDonell et al., 1986). However, when 16S rRNA sequences for all three *Edwardsiella* species present in the MicroSeq database (Applied Biosystems and Applera Corporation Business) were used to conduct a BLAST search (the results of which provide the top 20 related sequences ordered by minimum distance) and then subjected to a phylogenetic analysis based upon the neighbor-joining (NJ) method (Saitou and Nei, 1987), the closest neighbors in the family Enterobacteriaceae to the genus *Edwardsiella* were found to be *Trabulsiella guamensis* and *Enterobacter sakazakii* (Fig. 1).

The legitimacy of each *Edwardsiella* species, as determined by small-subunit rRNA sequences, is also supported by phylogenetic analysis of the superoxide dismutase (*sodB*) gene, which has two clusters (Yamada and Wakabayashi, 1999). Cluster 1 contains *E. ictaluri* and cluster 2 contains *E. hoshinae* and typical *E. tarda* strains.

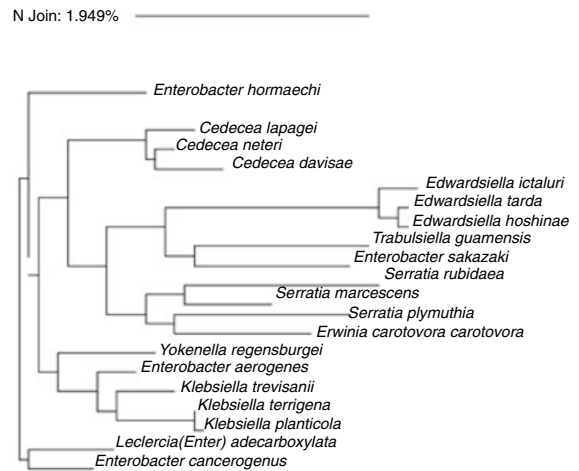


Fig. 1. Phylogenetic placement of *E. tarda* using 16S rDNA sequences analyzed by the neighbor-joining method. (From MicroSeq™ Analysis Software, 1999, with permission.)

## Habitat

Unfortunately, no extensive surveys on the natural habitat(s) of *Edwardsiella* species have ever been undertaken. Collective evidence, however, indicates freshwater environments and animals that have extensive contact with aquatic ecosystems are, most likely, the primary habitat of the edwardsiellae (Janda and Abbott, 1993). This hypothesis is supported by the low carriage rate of *E. tarda* in the human gastrointestinal tract (Janda and Abbott, 1993) and numerous reports describing the isolation of *Edwardsiella* from mammals, birds, reptiles, amphibians, and marine and freshwater life (Janda and Abbott, 1998). More specifically, as noted in Table 2, freshwater fish appear to be the primary habitat for at least two *Edwardsiella* species, *E. tarda* and *E. ictaluri*. *Edwardsiella ictaluri* is almost exclusively associated with ictalurid fish, although this species has on occasion been cultured from non-ictalurid fishes. *Edwardsiella tarda* has a broader host range amongst piscine species (Table 2). Other marine life from which *E. tarda* can occasionally be isolated includes mullet (*Mugil cephalus*), cultured sea bream (*Evynnis japonicus*), seals, sea lions, mussels and clams (Frerichs, 1989; Sakazaki and Tamura, 1991; Janda and Abbott, 1998). Both species also are recovered from environmental samples associated with aquatic ecosystems including pond water and sediment samples (Wyatt et al., 1979; Yamada and Wakabayashi, 1999).

In addition to fish, reptiles and amphibians also are likely reservoirs for *E. tarda* (Table 3). Reptiles found colonized or infected with *E. tarda* include alligators, crocodiles, lizards (e.g.,

Table 2. Isolation of *Edwardsiella* species from fish.

Organism	Common name	Scientific name	
<i>E. tarda</i>	Nile tilapia	<i>Oreochromis niloticus</i>	
	Ayu	<i>Plecoglossus altivelis</i>	
	Japanese flounder	<i>Paralichthys olivaceus</i>	
	Japanese eel	<i>Anguilla japonica</i>	
	Australian eel	<i>Anguilla reinhardtii</i>	
	Chinook salmon	<i>Oncorhynchus tshawytscha</i>	
	Largemouth bass	<i>Micropterus salmoides</i>	
	Rainbow trout	<i>Oncorhynchus mykiss</i> , <i>Salmo gardineri</i>	
	Tilapia	<i>Tilapia nilotica</i>	
	Ornamental fish	<i>Pterophyllum scalare</i> , <i>Betta splendens</i>	
	Zairese fish	<i>Ophiocephalus</i> , <i>Eutropius</i>	
	<i>E. ictaluri</i>	Channel catfish	<i>Ictalurus punctatus</i>
		White catfish	<i>Ictalurus catus</i>
		Brown bullhead	<i>Ictalurus nebulosus</i>
Walking catfish		<i>Clarias batrachus</i>	
Rhamphichthyid green knife fish		<i>Eigenmannia virescens</i>	
Cyprinad danio		<i>Danio devario</i>	
	Ornamental fish	<i>Puntius conchonius</i>	

Vandamme and Vandepitte, 1980; Hawke et al., 1981; Amandi et al., 1982; Vandepitte et al., 1983; Humphrey et al., 1986; Eaves et al., 1990; Baxa et al., 1990; Francis-Floyd et al., 1993; Reddacliff et al., 1996; and Yamada and Wakabayashi, 1999.

Table 3. Natural reservoirs of edwardsiellae.

Organism	Major reservoir	Incidental hosts
<i>E. tarda</i>	Amphibians	Mammals
	Fish	
	Reptiles	
<i>E. ictaluri</i>	Ictalurid fish	Nonictalurid fish
<i>E. hoshinae</i>	Unknown	Lizards
		Birds

skinks), tortoises, aquatic turtles and snakes, whereas amphibians found colonized include frogs and toads (Sakazaki and Tamura, 1991; Janda and Abbott, 1998). A Russian survey of zoo reptiles found 43% of crocodiles, 36% of snakes, 24% of tortoises, and 18% of lizards carried edwardsiellae (Kalina et al., 1981). Roggen-dorf and Mueller (1976) isolated *E. tarda* from 20% of tortoises, 12% of snakes and 3% of lizards they tested and suggested that tortoises might be a normal habitat for this bacterium. A study conducted on wild animals in Panama found 6% of toads and 5% of snakes harbored *E. tarda* (Kourany et al., 1977). Sharma (1979) found the intestinal tract of 21% of wild toads tested positive for *E. tarda*. Birds from which *E. tarda* has been isolated include bald eagles, blue herons, brown pelicans, gulls, king vultures, loons, ostriches, penguins and sandbill cranes (Janda and Abbott, 1998). After seagulls (*Larus*) were implicated in the contamination of fish in processing plants, Berg and Anderson (1972) hypothesized that birds might disseminate edwardsiellae from natural aquatic ecosystems to other mammals. Much less often, warm-

blooded mammals such as cattle, dogs, monkeys, opossums, panthers, pigs, rats and skunks have been found to transiently harbor *E. tarda* (Kourany et al., 1977; Sakazaki and Tamura, 1991; Janda and Abbott, 1998).

Little is known regarding the habitat of *E. hoshinae*. In the only publication on this species, *E. hoshinae* was isolated from three monitor lizards (*Varanus* sp.), two puffins (*Fratercula artica*), an unspecified lizard, a flamingo (*Phoenicopterus ruber*), and water (Grimont et al., 1980).

## Isolation

Although virtually indistinguishable from H<sub>2</sub>S-positive *Salmonella* on most enteric plating media, *E. tarda* can be readily isolated from humans and animals on enteric plating media containing fermentable substrates, such as lactose and sucrose (MacConkey, xylose-lysine-desoxycholate [XLD], Hektoen, *Salmonella-Shigella* [SS] and desoxycholate citrate [DC] agars). Colonies appear colorless and therefore assume the color of the plating medium. On those media that incorporate detectors of H<sub>2</sub>S (all of the above listed media except MacConkey), colonies will have a black center indicating H<sub>2</sub>S has been produced (Fig. 2).

Biogroup 1 strains of *E. tarda*, which are H<sub>2</sub>S-negative and sucrose-positive, will be most easily identified on media containing only lactose (MacConkey, SS and DC agars), and they will not have black centers. Bismuth sulfite and brilliant green agars, both excellent for *Salmonella*

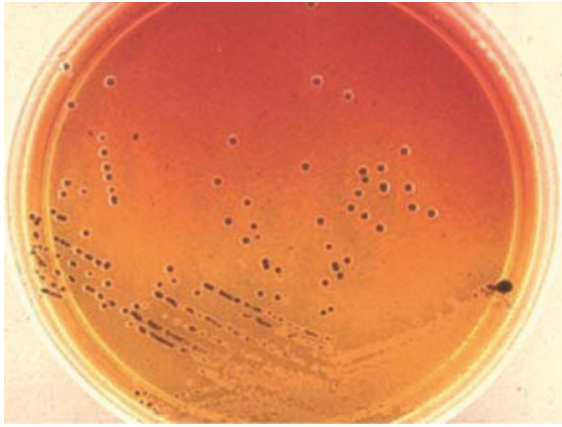


Fig. 2. Colorless colonies of *E. tarda* on *Salmonella-Shigella* (SS) agar showing H<sub>2</sub>S-positive centers.

isolation, may inhibit growth of *E. tarda* (Sakazaki and Tamura, 1991). In 1973, Muyembe et al. found intrinsic resistance to colistin among strains of *E. tarda*, thus incorporation of this polypeptide antibiotic into any enteric plating media should enhance the isolation of *E. tarda*. Despite several reports that indicate 10% of *E. tarda* strains are susceptible to colistin (Reinhardt et al., 1985; Waltman and Shotts, 1986a), the addition of this antibiotic to several novel media has (in fact) enhanced the isolation of *Edwardsiella*. Lindquist (1991) created a new isolation medium, *Edwardsiella tarda* (ET) agar, by adding colistin to MacConkey agar base.

#### ET Medium for Isolation of *E. tarda* (Lindquist, 1991)

MacConkey agar base	40.0 g
Yeast extract	1.0 g
Agar	4.5 g
Distilled water	100.0 ml

Autoclave and then cool; add the following two filter-sterilized solutions:

Glucose	2.0 g
Sucrose	5.0 g
Mannitol	5.0 g
Xylose	5.0 g
L-Lysine	10.0 g
Sodium thiosulfate	6.8 g
Ferric ammonium sulfate	0.8 g
Distilled water	100.0 ml
Colistin (1 mg/ml)	10.0 ml

Colonies of *E. tarda* appear clear to whitish with black centers after incubation at 37°C overnight. *Edwardsiella tarda* biogroup 1 strains, which ferment sucrose, grow on this medium as red colonies with black centers. Lindquist (1991) recovered *E. tarda* from 30.2% (16/53) of water samples on ET agar. All H<sub>2</sub>S-positive colonies were identified as *E. tarda*, whereas none of the H<sub>2</sub>S-negative colonies were *E. tarda*.

Inasmuch as *E. hoshinae* (like *E. tarda* biogroup 1 strains) ferments sucrose, MacConkey, SS and DC agars are the most suitable enteric plating media for isolation. Colonies will be small (1–2 mm); *E. hoshinae* strains will grow at either 30° or 37°C (Grimont et al., 1980). *Edwardsiella ictaluri* does not ferment lactose or sucrose, and despite its inability to produce H<sub>2</sub>S, it should be easily recognizable on enteric plating media. However, because it is slow growing, even at preferred temperatures of 25° to 30°C, requiring 48 to 72 h to form 1–2 mm colonies (Sakazaki and Tamura, 1991), it is often overgrown by other flora. Shotts and Waltman (1990) devised a selective medium called *E. ictaluri* medium (EIM) to specifically address this problem.

#### EIM of Waltman and Shotts (1989)

Bacto-peptone	10.00 g
Yeast extract	10.00 g
Agar	17.00 g
Phenylalanine	1.25 g
Ferric ammonium sulfate	1.20 g
Sodium chloride	5.00 g
Brom thymol blue	0.03 g
Bile salts	0.10 g
Distilled water	100.0 ml

Adjust the pH to 7.0–7.2, autoclave at 121°C for 15 minutes. Cool and add 10.0 ml of a filter-sterilized solution containing 3.5 g of mannitol and 10 mg of colistin just before pouring plates.

On EIM, *E. ictaluri* and *E. tarda* produce small (1 mm) green, translucent colonies after 48 hours, which are easily distinguishable from the few environmental flora that are not inhibited. Unfortunately, swarming *Proteus* species can still grow on this medium but their growth would be uninhibited on other media as well. Use of EIM agar has been found to enhance the isolation of *E. ictaluri* in channel catfish coinfecting with *Aeromonas hydrophila*, an organism that otherwise would easily overgrow *E. ictaluri* (Earlix et al., 1996).

*Edwardsiella* can be recovered from all enteric enrichment broths including selenite cysteine, Hajna's Gram-negative (GN) and tetrathionate broths. Van Damme and Vandepitte (1980) found that the recovery of *E. tarda* was better in tetrathionate broth without the addition of iodine. Alternative enrichment broths that have been used for edwardsiellae include double-strength SS broth (Wyatt et al., 1979) and strontium chloride B broth (Iveson, 1973).

#### Strontium Chloride B Broth (Iveson, 1973)

Bacto-peptone	0.5 g
Sodium chloride	0.8 g
Potassium dihydrogen phosphate	0.1 g
Strontium chloride (60% w/v)	6.0 ml
Distilled water	100.0 ml

Sterilize by steaming 30 minutes; adjust pH to 5.0–5.5.

Asymptomatic channel catfish carrying *E. ictaluri* that appear normal and are negative by conventional culture techniques can spread enteric septicemia to susceptible catfish populations. To detect *E. ictaluri* in these situations, an enzyme-linked immunosorbent assay (ELISA) using anti-*E. ictaluri* hybridoma supernatant 9G4-D9 antibody was developed (Earlix et al., 1996). Homogenized catfish tissue, treated with 0.5% Triton-X, was filtered onto a 0.45 micron pore nitrocellulose filter, which was subsequently placed on EIM agar and incubated 24 h at 25°C before ELISA testing.

## Identification

*Edwardsiella tarda* is an easily recognizable member of the Enterobacteriaceae. It produces H<sub>2</sub>S, decarboxylates lysine, produces indole from tryptophan (Fig. 3), and as indicated by its species designation *tarda*, does not ferment any carbohydrate or sugar alcohols except glucose, maltose and occasionally glycerol.

Biochemicals that separate *E. tarda* from other H<sub>2</sub>S-positive genera of the Enterobacteriaceae, many of which are associated with water habitats, can be found in Table 4.

Biogroup 1 strains of *E. tarda* ferment sucrose, mannitol and arabinose in addition to maltose but are H<sub>2</sub>S-negative. Two recent publications (Walton et al., 1993; Leung, 1996) have described sucrose-positive strains of *E. tarda* that are H<sub>2</sub>S-positive and do not ferment mannitol or arabinose, and therefore, do not belong to biogroup 1. It remains to be seen if these strains are a new species or a second biogroup of *E. tarda*.

Because *E. tarda* biogroup 1 strains, *E. hoshinae* and *E. ictaluri* all lack the ability to produce

H<sub>2</sub>S, they may be difficult to separate from other inactive genera within the Enterobacteriaceae. Their ability to decarboxylate lysine and ornithine and the inability to utilize citrate or deaminate phenylalanine are helpful distinguishing characteristics. They also may easily be confused with inactive strains of *E. coli*; however, the colistin or polymyxin B resistance of *Edwardsiella* can be used to separate them from inactive strains of *E. coli*. *Edwardsiella ictaluri* cultures should be incubated at 25°C, the temperature at which they have increased biochemical activity. For instance, motility that is present at 25°C may be delayed 3 to 5 days, or be negative at 37°C. Early descriptions of this organism by Hawke (1979) indicated gas was not produced from

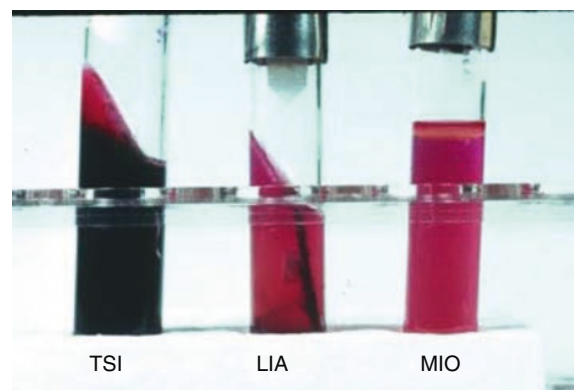


Fig. 3. Typical reactions for *E. tarda* in triple sugar iron (TSI), lysine iron (LIA), and motility-indole-ornithine (MIO) agars. Both TSI and LIA show H<sub>2</sub>S production (blackening); the purple color in the butts of the LIA and MIO tubes indicate lysine and ornithine were decarboxylated. Motility is exhibited as turbidity throughout the MIO tube and the pink color in the Kovac's reagent at the top indicates indole production.

Table 4. Separation of *E. tarda* from other H<sub>2</sub>S-positive Enterobacteriaceae.

Organism	Indole <sup>a</sup>	Lysine <sup>b</sup>	Citrate <sup>c</sup>	PPA	Acid from	
					Rhamnose	Arabinose
<i>E. tarda</i>	+	+	-	-	-	-
<i>Budvicia aquatica</i>	-	-	-	-	+	V
H <sub>2</sub> S <sup>+</sup> <i>Citrobacter</i> species	V	-	V	-	+	+
<i>Leminorella</i> species	-	-	V	-	-	+
<i>Proteus</i> species	V	-	V	+	-	-
<i>Pragia fontium</i>	-	-	V	V	-	-
Non-typhoidal <i>Salmonella</i>	-	+	+	-	+	+
<i>Trabulsiella guamensis</i>	V	+	V	-	+	+
<i>Morganella morganii</i> <sup>d</sup>	V	V	-	+	-	-

Symbols: +, ≥90%; -, ≤10%; and V, 11–89%.

Abbreviations: PPA, phenylalanine deaminase; H<sub>2</sub>S<sup>+</sup>, H<sub>2</sub>S-positive.

<sup>a</sup>Indole production is from tryptophan.

<sup>b</sup>Lysine decarboxylation.

<sup>c</sup>Utilization of citrate as sole carbon source.

<sup>d</sup>About 20% of *M. morganii* ss *morganii* are H<sub>2</sub>S-positive.

Data from Farmer (1999).

Table 5. Biochemical characteristics used to separate *edwardsiellae*.

Organism	Indole <sup>a</sup>	H <sub>2</sub> S	Malonate <sup>b</sup>	Acid from			
				Sucrose	Mannitol	Arabinose	Trehalose
<i>E. tarda</i>	+	+	-	-	-	-	-
<i>E. tarda</i> biogrp 1	+	-	-	+	+	+	-
<i>E. ictaluri</i>	-	-	-	-	-	-	-
<i>E. hoshinae</i>	V	-	+	+	+	V	+

Symbols: +, ≥90%; -, ≤10%; and V, 11–89%.

<sup>a</sup>Indole production from tryptophan.

<sup>b</sup>Utilization of malonate as a sole carbon source.

Data from Farmer (1999).

Table 6. Tests results that are the same in all *Edwardsiella*.

<i>Edwardsiella</i> are:		
Positive	Negative	Variable
Catalase	Voges-Proskauer	Gas from glucose:
Nitrate reduction	<i>o</i> -Nitrophenyl-β-D-galactosidase	100% <i>E. tarda</i>
Lysine decarboxylase	Urease	50% <i>E. tarda</i> biogrp 1
Acid production from:	Oxidase	35% <i>E. hoshinae</i>
Glucose	Arginine dihydrolase	50% <i>E. ictaluri</i>
	Sodium acetate utilization	
	Mucate utilization	Citrate utilization:
	Esculin	1% <i>E. tarda</i>
	Gelatinase	0% All other species
	Phenylalanine deaminase	
	Acid production from:	Acid production from:
	Lactose	Salicin
	Adonitol	50% <i>E. hoshinae</i>
	Dulcitol	0% All other species
	Inositol	Glycerol
	Sorbitol	30% <i>E. tarda</i>
	Raffinose	65% <i>E. hoshinae</i>
	Rhamnose	0% Other species
	Xylose	
	Cellobiose	
	α-Methyl-D-glucoside	
	Erythritol	
	Melibiose	
	Arabitol	

Adapted from Farmer, 1999; Waltman et al., 1986a; Waltman et al., 1986b; and Sakazaki and Tamura, 1991.

glucose at 37°C; however, Waltman and Shotts (1986a) found diminished gas production in 54% of their strains at 37°C. *Edwardsiella* species are easily differentiated from each other by a few simple biochemical reactions (Table 5).

Biochemical characteristics uniformly positive or negative for all *Edwardsiella* are included in Table 6. Additionally, tests for which some variability has been noted amongst species are also listed.

Both *E. tarda* and *E. ictaluri* lack proteases, lipases, esterases, pectinase, collagenase, alginate, chitinase and hyaluronidase. However, 100% and 56% of 100 strains of both *E. ictaluri* and *E. tarda*, respectively, degraded chondroitin sulfate (Waltman et al., 1986b; Waltman et al., 1986c). Information regarding the production of these enzymes was not found for *E. hoshinae*.

## Serotyping

Because *E. tarda* strains are biochemically homogeneous and relatively inactive, there are no biotyping systems. Consequently, serotyping has been the method used to epidemiologically define strains. Two serotyping schemes for *E. tarda*, developed independently in Japan and the United States, were merged in 1988 to form a single international scheme (Tamura et al., 1988). The scheme is now comprised of 61 somatic (O) and 45 flagellar (H) antigens, and serotyping is performed as described by Sakazaki (1984). Briefly, O antigens are determined by slide agglutination assay using equal amounts of boiled saline suspension from an overnight agar slant culture and working dilution of O antisera. The H antigens are identified by tube agglu-



tionation using an overnight culture grown in brain heart infusion broth to which an equal volume of saline with 0.2% thimerosal has been added. Unlike *Salmonella*'s, *E. tarda*'s flagellar antigens are monophasic and do not occur in complexes. Serotyping is now performed only at the National Institute of Health, Tokyo, Japan (Tamura et al., 1988). There are no serotyping schemes for *E. ictaluri* and *E. hoshinae*.

Strains of *Edwardsiella* also may be epidemiologically typed by plasmid analysis (Reger et al., 1993). The utility of ribotyping, PCR typing, or pulsed-field gel electrophoresis for classifying these strains has not been reported.

## Antimicrobial Susceptibility

All species of *Edwardsiella* are uniformly susceptible to all antibiotics or to all  $\beta$ -lactam

antibiotic- $\beta$ -lactamase inhibitor combinations (Table 7) that are used to treat gastrointestinal and systemic infections (Reinhardt et al., 1985; Reger et al., 1993; Clark et al., 1991). Interestingly, this includes the  $\beta$ -lactam antibiotics even though all isolates of *E. tarda* tested to date have been found to produce  $\beta$ -lactamases. None of thirteen *E. hoshinae* or ten *E. ictaluri* strains tested by Reger et al. (1993) possessed  $\beta$ -lactamases. Ninety percent of *E. tarda* strains (Waltman and Shotts, 1986a) and all *E. ictaluri* strains studied by Shotts and Waltman (1990) are resistant to colistin or polymyxin B, whereas *E. hoshinae* are variable in their susceptibility to these agents (Sakazaki and Tamura, 1991). Because of their slow-growth, *E. ictaluri* require 48 h of incubation at 25°C for broth dilution (Reger et al., 1993) and disk diffusion (Sakazaki and Tamura, 1991) testing. The use of only four disks per 150-mm Mueller-Hinton agar plate is

Table 7. MIC<sub>90</sub> of Antimicrobials with *Edwardsiella* species.

Antimicrobial	MIC <sub>90</sub>				
	<i>E. tarda</i>		<i>E. ictaluri</i>	<i>E. hoshinae</i>	
	(1)	(2)	(3)	(3)	(3)
Amikacin	4	—	—	—	—
Gentamicin	1	2.0	0.625	0.31	0.156
Tobramycin	1	—	—	—	—
Ampicillin	0.25	0.5	0.1	0.156	0.1
Ampicillin-sulbactam	0.25/0.12	—	—	—	—
Amoxicillin	1	—	—	—	—
Amoxicillin-clavulanate	1.0/0.5	—	—	—	—
Cephalothin	2	—	—	—	—
Cefoxitin	0.5	—	—	—	—
Cefamandole	≤0.06	—	—	—	—
Ceftriaxone	≤0.06	—	—	—	—
Cefotaxime	≤0.06	≤0.063	0.01	0.01	0.001
Ceftazidime	0.25	—	—	—	—
Cefoperazone	≤0.06	—	—	—	—
Cefoperazone-sulbactam	≤0.06/0.03	—	—	—	—
Piperacillin	0.5	—	0.1	0.156	0.1
Piperacillin-tazobactam	0.25/0.06	—	—	—	—
Ticarcillin	8	—	—	—	—
Ticarcillin-clavulanate	4/2	—	—	—	—
Imipenem	0.25	—	0.156	0.31	0.1
Aztreonam	≤0.06	—	0.012	0.01	0.1
Ciprofloxacin	≤0.06	≤0.063	0.0001	0.0001	0.01
Enoxacin	—	0.125	—	—	—
Norfloxacin	—	≤0.063	—	—	—
Chloramphenicol	—	1.0	—	—	—
Nalidixic acid	—	2.0	—	—	—
Tetracycline	—	1.0	—	—	—
Doxycycline	—	—	0.31	0.156	0.156
Trimethoprim	—	0.25	—	—	—
Sulfamethoxazole	—	38	—	—	—
Trimethoprim-sulfamethoxazole	—	0.13/2.4	—	—	—

Symbol: —,

Abbreviation: MIC<sub>90</sub>, minimum inhibitory concentration of 90% of isolates.

Adapted from 1) Clark et al., 1991; 2) Reinhardt et al., 1985; and 3) Reger et al., 1993.

recommended because the zones of growth inhibition are large.

## Genetics

### Plasmids

Extrachromosomal elements have been detected in all three *Edwardsiella* species. Virtually all *E. ictaluri* are known to carry small plasmids <10 kb in size. Lobb and Rhoades (1987) found cryptic plasmids of 5.7 and 4.9 kb in all 18 *E. ictaluri* strains recovered from channel catfish, although their relative mobility in agarose gels and their restriction maps indicated the plasmids were not closely related to each other. Common endonucleases found to cleave both plasmids included *EcoRI*, *BglI*, and *PstI*. Subsequent studies have indicated that most *E. ictaluri* strains, regardless of origin, contain classes of homologous plasmids of similar, but not identical, sizes that are genetically related (Reid and Boyle, 1989; Lobb et al., 1993). These plasmid classes include a 4.0 to 4.7 kb plasmid and a 5.6 kb plasmid (Reger et al., 1993). This suggests that these cryptic plasmids are extremely stable and highly conserved in almost all *E. ictaluri* isolates. One isolate of *E. ictaluri* from a green knife fish was found to contain four plasmids ranging in size from 3.1 to 6.0 kb (Lobb et al., 1993). Minor variations in plasmid carriage, other than the two major classes, have been noted in strains recovered from non-ictalurid fish (Reid and Boyle, 1989).

In contrast to the small-size plasmids detected in *E. ictaluri* strains, much larger extrachromosomal elements have been isolated from both *E. tarda* and *E. hoshinae* isolates. Plasmids in these two species range in molecular mass from 2 to 120 Mda, and unlike in *E. ictaluri*, plasmid carriage appears to vary significantly from strain to strain (Janda et al., 1991a). Reger et al. (1993) found that 6 of 13 *E. hoshinae* strains contained a single plasmid of ~54 kb, while approximately half of the *E. tarda* strains tested carried plasmids ranging from 5.0 to 76 kb. Functions for these plasmids have not been identified to date. The R plasmids encoding resistance to both tetracycline and sulfonamide, or to the combination of chloramphenicol, tetracycline and sulfonamide have been detected in strains of *E. tarda* recovered from eel ponds (Aoki, 1988). These R plasmids have been classified into incompatibility groups A–C.

### Transformation

*Edwardsiella* species have been transformed with both plasmids and transposons. *Escherichia coli* can transfer R plasmids to *Edwardsiella icta-*

*luri* strains at frequencies ranging from  $10^{-3}$  to  $10^{-6.5}$  (Waltman et al., 1989). Resistance markers transferred on a ~40 MDa plasmid included tetracycline, chloramphenicol, kanamycin, ampicillin and trimethoprim-sulfamethoxazole. Similarly, one strain of *E. ictaluri* was shown to be capable of transferring R plasmids to both *E. coli* and *Yersinia ruckeri* at frequencies approaching  $10^{-4}$ . *Edwardsiella tarda* strains have been stably transformed with plasmids encoding green or blue fluorescent protein (Ling et al., 2000). Strauss et al. (1997) was able to mutagenize a strain of *E. tarda* by transformation with a plasmid containing an ampicillin-resistance gene and a transposon Tn5 derivative with a tetracycline resistance gene. However, Strauss noted that the transformation frequency of *E. tarda* was ~1,000-fold less than that of *E. coli*.

## Ecology

The microecology of *Edwardsiella* species, and in particular *E. tarda*, is poorly understood. At present it is unclear whether most *Edwardsiella* species are obligate pathogens or can thrive in environments outside of their primary hosts (Thune et al., 1993). Data collected on known sources of *E. tarda* suggest that this species does not exist as a free-living microorganism in natural waters. Most aquatic isolates of *E. tarda* originate from pond or culture water inhabited by various fish, eels, amphibians, etc., that harbor edwardsiellae in their digestive tracts or on their exterior surfaces. Warmer temperatures during summer months are known to increase incidence of recovery of *E. tarda* from catfish pond water and mud samples (Wyatt et al., 1979). In their study, King and Shotts (1988) found that strains of *E. tarda* survived poorly in saline suspensions; viable numbers declined by >100-fold within 72 h. However, cocultivation of *E. tarda* with a ciliated protozoan, *Tetrahymena pyriformis*, yielded a 2–3 fold increase in bacterial numbers over the initial inoculum during the same time interval (King and Shotts, 1988). This suggests that persistence of *E. tarda* in freshwater environments may be facilitated by the ingestion of edwardsiellae by ciliated protozoans, leading to subsequent intracellular multiplication of bacteria within the host. Thus protozoa may play an intermediary role in the *E. tarda* colonization of other animals within aquatic ecosystems. The ecologic distribution of *E. ictaluri* appears restricted probably because it is almost exclusively an obligate pathogen of ictalurid fish (Hawke et al., 1981) and has an optimal lower growth temperature of 25°C (see Physiology section). Studies conducted with channel catfish fingerlings indicate that mortality increases in *E.*

*ictaluri*-infected fish in water temperatures ranging from 23 to 28°C (Francis-Floyd et al., 1987). The fact that *E. hoshinae* has not been isolated from any sources since its initial taxonomic description indicates that either this species has an extremely limited ecologic distribution or is uniquely adapted to an as yet unknown host.

## Epidemiology

### Fish Diseases

Factors regulating the infectivity of *Edwardsiella* in fish are probably similar, if not identical, to those associated with piscine diseases caused by other aquatic pathogens such as *Aeromonas*. It is well recognized that, for both *E. ictaluri*- and *E. tarda*-associated infections, morbidity and mortality increases as water temperatures rise (Francis-Floyd et al., 1987; Greenlees et al., 1998). Other factors that may increase the incidence of infections include stress and organic load (Greenlees et al., 1998).

### Human Illnesses

Although human infection caused by *E. tarda* is a relatively infrequent event, there is surprisingly good information in the literature collected from individual case reports on risk factors associated with edwardsiellosis. Most cases of gastroenteri-

tis are associated with handling or consumption of raw or improperly cooked fish. Contact with unusual or exotic pets, although less common, is also a source of infection. Most pet-related cases of *Edwardsiella gastroenteritis* involved contact with or handling turtles (Marsh and Gorbach, 1982; Nagel et al., 1982). However, other pets, including reptiles, have been implicated in transmission (Fang et al., 1991). Cases of *E. tarda* septicemia most often occur in immunocompromised persons. The most common underlying risk factor in such individuals is hepatic cirrhosis; ~50% of persons developing *E. tarda* bacteremia have liver-related disease such as hepatosplenomegaly, icterus or hepatoma (Janda and Abbott, 1993). Wound infections typically result from penetrating or blunt trauma to mucosal surfaces exposed to either aquatic environments or inhabitants. Table 8 lists the main risk factors associated with *E. tarda* infections.

## Disease Manifestations

### Fish Diseases

As noted in Table 9, both *E. ictaluri* and *E. tarda* are important pathogens of fish (Frerichs, 1985; Thune et al., 1993). The causative agent of enteric septicemia of catfish (ESC; Fig. 4), *E. ictaluri* is responsible for enormous economic losses to the channel catfish industry, the largest aquaculture business in the United States (Hawke et al., 1981). A 1988 fish mortality summary for catfish listed *E. ictaluri* (n = 1,169) as the leading cause of death, accounting for 48% of all cases reported (Durborow et al., 1991). Typically occurring in the spring to early fall when water temperatures are between 22–28°C (Thune et al., 1993), ESC has two forms, either a rapidly fatal, acute gastrointestinal septicemia or a chronic condition typified by a “hole in the head” lesion, associated with a lower mortality rate (Thune et al., 1993).

Characteristics of ESC include gross petechial hemorrhagic lesions occurring around the throat and mouth, cutaneous lesions on the lateral body surface, and pale gills (Hawke, 1979). Internally,

Table 8. Risk factors associated with *Edwardsiella* infections.

Disease or condition	Risk factor or epidemiologic association
Gastroenteritis	Consumption of raw or undercooked fish Contact with exotic pets
Septicemia	Underlying liver disease Preceding episode of <i>E. tarda</i> diarrhea Occupational (zoo) or environmental exposure
Wound infections	Catfish-related injuries Recreational/occupational activities related to aquatic exposure (e.g., swimming, fishing)

Table 9. Diseases of fish and other animals caused by *Edwardsiella* species.

Organism	Host	Disease	
		Type	Illness
<i>E. ictaluri</i>	Channel catfish	Enzootic, Epizootic	Enteric septicemia of catfish
<i>E. tarda</i>	Channel catfish	Epizootic	Emphysematous putrefactive disease
<i>E. tarda</i>	Largemouth bass	Epizootic	Septicemia; deep cutaneous ulcers
<i>E. tarda</i>	Striped bass	Epizootic	Hemorrhagic ulcers
<i>E. tarda</i>	Rainbow trout	Sporadic	Septicemia
<i>E. tarda</i>	Rockhopper penguins	Sporadic	Enteritis
<i>E. tarda</i>	Reptiles	Sporadic	Gastrointestinal disease

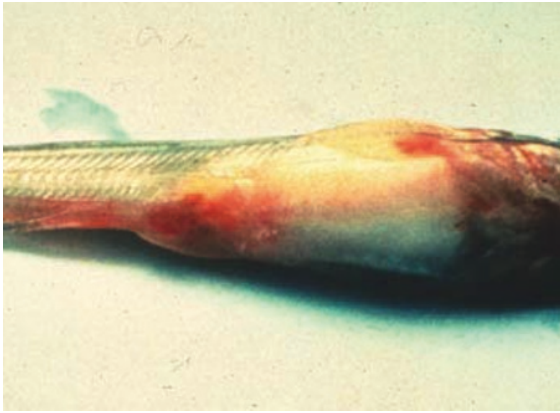


Fig. 4. Typical external appearance of catfish with enteric septicemia. (Courtesy of John Plumb, Department of Fisheries and Allied Aquaculture, Auburn University, Auburn, AL.)

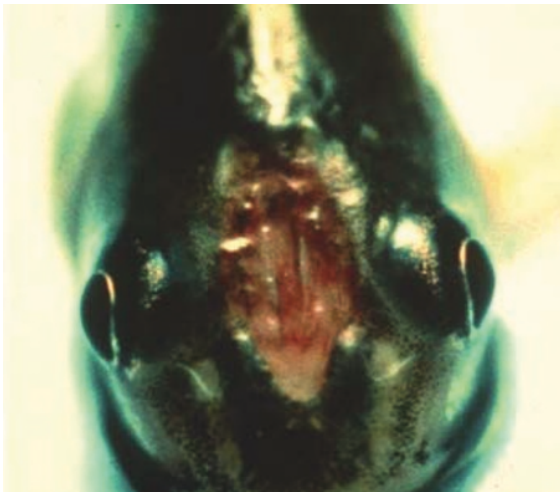


Fig. 5. "Hole-in-the-head" lesion in a fingerling channel catfish. (Courtesy of Emmett Shotts, National Fish Health Research Laboratory, Kearneysville, WV.)

fish suffering from ESC exhibit renal hypertrophy, hemorrhage and necrosis in the liver, bloody ascites fluid, and widespread petechial hemorrhages in inner walls of the visceral cavity (Hawke, 1979). The "hole-in-the-head" lesion occurs on the frontal bone of the skull of channel catfish (Fig. 5), and involves erosion of the skin and muscle overlying the skull (Francis-Floyd et al., 1987). *Edwardsiella tarda* also is recognized as an occasional pathogen of channel catfish, largemouth bass, and several other freshwater species including rainbow trout (Meyer and Bulloch, 1973; Amandi et al., 1982; Francis-Floyd et al., 1993; Reddacliff et al., 1996).

In catfish infected with *E. tarda*, Meyer and Bulloch (1973) described "emphysematous putrefactive disease" as cutaneous lesions on the

lateral surfaces of fish that develop into deep cavitory abscesses containing gas and necrotic material. In the Chesapeake Bay in 1994, a large *E. tarda*-associated epizootic in wild striped bass occurred that was typified by numerous irregular, hemorrhagic ulcers on the body and fins of the fish (Baya et al., 1997). Wild-type (classical) *E. tarda* was recovered in pure culture from the internal organs of striped bass including spleen, liver, and pronephroi and coelomic fluids (Baya et al., 1997). The role of *E. tarda* as an opportunistic pathogen in diseased chinook salmon (*Oncorhynchus tshawytscha*) and steelhead trout in the Pacific Northwest (Amandi et al., 1982) was also suspected.

### Diseases of Other Animals

Particularly in zoological parks, *E. tarda* has been known to cause sporadic infections in a variety of animals. In 1985, an outbreak of enteritis in Rockhopper penguins, in which eight birds died, was attributed to *E. tarda* (Cook and Tappe, 1985). Histologic analysis of the small intestine of four of these birds revealed segmental-to-diffuse enteritis with villous atrophy, goblet-cell hyperplasia, and lymphoplasmacytic infiltrates in the lamina propria. Necropsy specimens on four of five penguins submitted for bacteriologic analysis grew *E. tarda*. A retrospective review of mortality between 1973–83 at the Detroit zoo found that bacteria caused ~37% of reptilian deaths (Kaneene et al., 1985). *Edwardsiella* was one of five bacterial genera (that also included *Aeromonas*, *Salmonella*, *Proteus* and *Pseudomonas*) responsible for most of the mortality that occurred primarily in iguanas and snakes.

### Human Infections

An uncommon human pathogen, *E. tarda* is the only species in its genus known to cause disease in man (Janda and Abbott, 1993). Categories of illnesses associated with *E. tarda* infection, in decreasing order of frequency, include gastroenteritis, septicemia, wound infections, hepatic abscesses and miscellaneous illnesses (Janda and Abbott, 1993).

Gastroenteritis, the most common *E. tarda*-associated disease in humans, has a worldwide distribution, although higher infection rates appear to occur in tropical and subtropical climates (Gilman et al., 1971; Kourany et al., 1971; Ovartlarnporn et al., 1986). Reasons for this demographic association include different dietary habits (high consumption of fish, often raw), increased fishing-related activities, or drinking water from freshwater sources that are in intimate contact with reptiles and amphibians (Kourany et al., 1977; Van Damme and Vande-

pitte, 1980). Infection rates in symptomatic persons with *E. tarda*-associated diarrhea have ranged from 0.2 to 13.9% in selected studies; carrier rates in these surveys vary from 0 to 5.4% (Janda and Abbott, 1993; Janda and Abbott, 1998). A Panamanian survey conducted between 1965 and 1972 found 1.1% of all non-urban persons were either colonized or infected with *E. tarda*, whereas none of the more than 2,700 urban dwellers surveyed carried this agent (Kourany et al., 1977). Similarly, in a study of the jungle-dwelling Orang Asli (Aborigines) of Western Malaysia, 30% of patients hospitalized with bloody diarrhea had stools containing *E. tarda* (Gilman et al., 1971). However, simultaneous recovery of *Entamoeba histolytica* from the same stools of many of these patients suggested chronic exposure to multiple enteric pathogens in their environment.

Other than a cluster of eight isolates recovered from asymptomatic persons (seven children and one teacher) associated with a Florida day-care center (Desenclos et al., 1990), no outbreak of *E. tarda* diarrhea has ever been reported in the United States. However, several lines of evidence suggest that some strains of *E. tarda* can cause diarrhea: 1) isolation rates are higher in symptomatic persons than in controls, 2) antibodies to *E. tarda* are present in serum, 3) edwardsiellae can be isolated from both the stool and blood of persons with gastroenteritis and septicemia, and 4) enteropathogenic mechanisms (see Pathogenicity) can be identified for select *E. tarda* isolates (Gilman et al., 1971; Janda and Abbott, 1993; Janda and Abbott, 1998). *Edwardsiella* gastroenteritis is usually a self-limiting infection. Several forms of *E. tarda*-associated gastroenteritis have been described including enteric fever, secretory enteritis and a dysenteric (bloody) form (Janda and Abbott, 1993). Secretory diarrhea is the most common form of illness with a low-grade fever and watery stools (5 bowel movements/day) as the predominant symptoms. Documented sources of exposure leading to *E. tarda* gastroenteritis include handling amphibians or possessing ornamental aquariums (Nagel et al., 1982; Marsh and Gorbach, 1982; Vandepitte et al., 1983).

Septicemia associated with *E. tarda* is rare. Although one report states that over 300 cases have been published (Peyrade et al., 1997), probably only 25 to 30 cases of *E. tarda* bacteremia are well described in the medical literature. Though *E. tarda* septicemia is an uncommon illness, the mortality rate associated with infection approaches 50% (Janda and Abbott, 1993). Patients typically present with fever and less often (36–50%) with diarrhea, chills, hypotension, epigastric pain, nausea and vomiting (Janda and Abbott, 1993; Janda and Abbott, 1998).

Approximately three-quarters of persons developing *E. tarda* sepsis are immunocompromised or have one or more other serious underlying conditions. Common maladies include pre-existing hepatobiliary disease, malignancy, diabetes mellitus and sickle cell disease (Jaruratanasirikul and Kalnauwakul, 1991; Janda and Abbott, 1993; Wu et al., 1995; Matsushima et al., 1996; Peyrade et al., 1997; Osiri et al., 1997). Recently, Schoenfeld et al. (1995) described a case of bacteremia in a 71-year-old man with chronic autoimmune thyroiditis whose blood and urine was positive for *E. tarda*; a source for this unusual infection was not identified. He was treated with gentamicin/mezlocillin, to which he responded favorably. Janda and Abbott (1993) noted that many persons presenting with *E. tarda* septicemia have underlying conditions associated with iron overload. These conditions include red cell sickling, neonatal jaundice, hepatic cirrhosis and leukemia. Matsushima et al. (1996) reported the first case of *E. tarda* septicemia associated with necrotizing fasciitis. This fatal infection in a 67-year-old Japanese man with liver cirrhosis and hepatoma, developed 15 days after his initial hospitalization. His right leg became edematous with hemorrhagic bullae; both blood and material from the bullae yielded *E. tarda*. Laboratory findings suggested that he died of disseminated intravascular coagulation. *Edwardsiella* bacteremia (complicated by uterine pyomyoma) was recently described in a 46-year-old aboriginal woman (Yang and Wang, 1999). This case of uterine pyomyoma, a rarely reported condition, was believed to have occurred via hematogenous seeding of bacteria on the large infarcted uterine myoma. Interestingly, the onset of her symptoms appeared a few hours after consuming three bottles of wine. Other conditions recently associated with *Edwardsiella* septicemia include septic arthritis and cellulitis (Osiri et al., 1997; Fournier et al., 1997). Although the initial sources of infection for most cases of *Edwardsiella* septicemia are unknown, one report implicated contaminated seafood as the most likely vehicle for their case (Wu et al., 1995). Recently, Nettles and Sexton (1997) described a case of prosthetic valve endocarditis caused by *E. tarda*. The patient, a 41-year-old man with AIDS, presented with diarrhea, fever and shortness of breath. Transesophageal echocardiogram revealed multiple oscillating masses on the mitral prosthetic valve and two sets of blood cultures grew *E. tarda*. His recent medical history was significant for injuring his hand on the barb of a catfish before consuming it, cooked medium rare.

This organism is also occasionally recovered from wounds, but its role in pathology is often unclear because these infections are often

polymicrobial. As with monomicrobial cases of septicemia caused by edwardsiellae, there are very few good, documented reports describing *E. tarda* as a bona fide wound pathogen. The medical history of persons infected with *E. tarda* often reveals exposure to freshwater environments or activities (swimming, diving and fishing). Penetrating injuries from catfish spines are the most common precipitating factor leading to wounds infected with *E. tarda* (Hargreaves and Lucey, 1990; Murphey et al., 1992; Banks, 1992; Ashford et al., 1998), and these usually occur on the extremities. Ashford et al. (1998) reported a case of *E. tarda* septic arthritis in a 20-year-old professional angler that developed after the pectoral-fin spine of a Lake Argyle or Ord River catfish (*Arius midgleyi*) punctured his knee. He underwent repeated arthrotomies, pulse lavage, drainage and treatment with multiple chemotherapeutic agents (flucloxacillin, gentamicin) before his infection resolved. Occasionally, coinfections of *E. tarda* with other aquatic microbial species such as *Aeromonas hydrophila* are reported (Vartian and Septimus, 1990; Hargreaves and Lucey, 1990; Murphey et al., 1992). These infections can vary dramatically from mild cellulitis to life-threatening soft tissue infections including gas gangrene (Hargreaves and Lucey, 1990; Janda and Abbott, 1998).

Abscesses, another type of wound infection involving the collection of pus in a cavity, rarely involve *E. tarda*. However, eight cases of *E. tarda* associated with hepatic abscesses have been reported (Kourany et al., 1977; Zigelboim et al., 1992; Janda and Abbott, 1998). Patients with these abscesses commonly present with fever accompanied by abdominal or right quadrant pain; most of these illnesses have occurred in healthy persons with no known risk factors (Janda and Abbott, 1998). The mortality rate associated with this disease is ~40%. Other rare wound infections associated with *E. tarda* include a tubo-ovarian abscess in a 42-year-old woman who had recently eaten poki (raw fish) just before developing symptoms suggestive of acute appendicitis (Pien and Jackson, 1995).

Other body fluids from which *E. tarda* has been isolated include bile and urine (Tan et al., 1977). The significance of many of these isolates cannot be determined because of a lack of clinical information.

## Pathogenicity

The study of which factors or virulence determinants regulate the ability of both *E. ictaluri* and *E. tarda* to cause disease in fish, humans and other animals is in its infancy. Waltman et al. (1986) have described two enzymatic activities

with pathogenic potential in strains of *E. ictaluri*. All strains (n = 100) produced a chondroitinase and ~97% produced a hemolysin. The chondroitinase may conceivably be involved in the formation of chronic "hole-in-the-head" lesions (Fig. 5) by degradation of native cartilage (Shotts et al., 1986; Thune et al., 1993). The  $\beta$ -hemolysin, a cytolytic protein, may be responsible for some of the hemodynamic changes (hematocrit, hemoglobin, plasma protein and glucose) associated with *E. ictaluri*-infected fish (Thune et al., 1993). The ulcerative lesions associated with ESC (e.g., petechial hemorrhages) might also be partially due to damage and lysis of host cells by direct contact with  $\beta$ -hemolysin (Fig. 4). Besides these two activities, *E. ictaluri* strains are refractory to the bactericidal effects of fresh human serum (Janda et al., 1991a; Reger et al., 1993). Resistance to complement-mediated lysis might facilitate the rapid invasion and migration of edwardsiellae from the intestine into internal organs during a fulminant episode of ESC (Thune et al., 1993). Adherence to tissue in the initial stages of infection may be facilitated by mannose-sensitive or mannose-resistant hemagglutinins acting as non-fimbrial adhesins (Wong et al., 1989; Thune et al., 1993). Strains of *E. ictaluri* do not possess significant amounts of glycocalyx, which might aid in adherence, on their cell surfaces as determined by electron microscopic analysis of ruthenium-stained cells (Wong et al., 1989).

There are several candidate virulence factors present in *E. tarda* that might play key roles in the development of diarrhea. Because *E. tarda* can cause both a secretory and bloody form of gastroenteritis, it is not surprising that potential factors operative in each type of infection have been identified. Marques et al. (1984) found *E. tarda* strains could invade HeLa cells (an epithelial cell line derived from a human adenocarcinoma), although all 12 isolates were negative in the keratoconjunctivitis (Sereacuteny test) assay. Janda et al. (1991b) confirmed the invasive ability of *E. tarda* and *E. hoshinae* (Fig. 6) in HEp-2 cells (a line derived from a human hepatocellular carcinoma; Fig. 7); these authors also detected the expression of a strong cell-associated  $\beta$ -hemolysin in *E. tarda* isolates.

The ability of *E. tarda* strains to invade non-professional phagocytes might be related to its ability to cause bloody diarrhea. Fish isolates of *E. tarda* invade epithelioma papillosum of carp (EPC) cells in vitro, suggesting that some of the invasive disease seen in piscine species caused by this microbe may be related to this property (Ling et al., 2000). Invasion appears to be a microfilament- and protein tyrosine kinase-dependent process as both cytochalasin D and genistein inhibit this process (Janda et al.,

Fig. 6. The adherence of *E. tarda* to HEp2 cells (A) magnification  $\cdot$  4,500; (B) single bacteria in membrane-bound vesicles 30 min postinfection, magnification  $\cdot$  15,000; (C) 2.5 h postinfection, magnification  $\cdot$  4,500 (D) 3.5 h postinfection, magnification  $\cdot$  3,000. (From Strauss et al., 1997, with permission.)

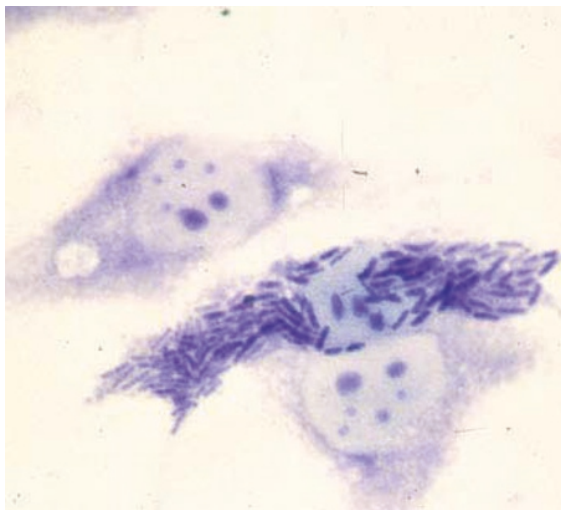
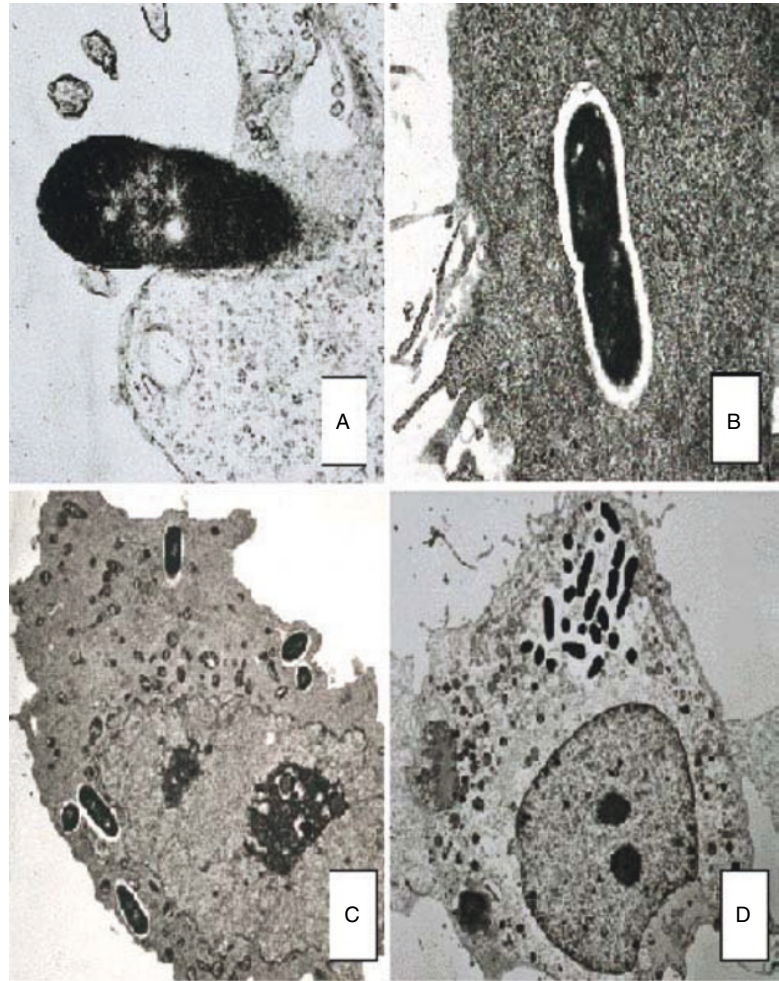


Fig. 7. HEp2 cells invaded by *E. tarda*, magnification  $\cdot$  200.

1991b; Ling et al., 2000). Subsequently, two  $\beta$ -hemolysins from *E. tarda* have been cloned and sequenced. The first, cloned from strain ET16, resides in an open-reading frame (ORF) of  $\sim$ 333

bp encoding for a 311 amino acid polypeptide with a molecular mass of 34 kDa (Chen et al., 1996). The structural gene for this  $\beta$ -hemolysin shares over 90% homology to the hemolysin BL of *Bacillus cereus* (Hirono et al., 1997). The frequency of this gene in *E. tarda* isolates is unknown. A second  $\beta$ -hemolysin from *E. tarda* strain MZ8901 has been cloned and sequenced by Hirono et al. (1997). The gene, designated *ethA*, resides in an ORF of 4,782 bp that encodes for a 165.3 kDa product. The deduced amino acid sequence of EthA shares the highest degree of homology (47%) with the Sh1A hemolysin of *Serratia marcescens* (Hirono et al., 1997). All 72 *E. tarda* strains tested reacted with an *ethA* probe, indicating that this appears to be the predominant  $\beta$ -hemolysin carried by most isolates. A study by Strauss et al. (1997) found that a transposon-mutagenized strain of *E. tarda* is noncytotoxic and defective for hemolysin production (Fig. 8) and is also unable to enter Hep-2 cells. This hemolysin appears to be very similar or identical to that described by Hirono et al. (1997). This implies that both hemolytic and invasive capabilities of edwardsiellae are linked

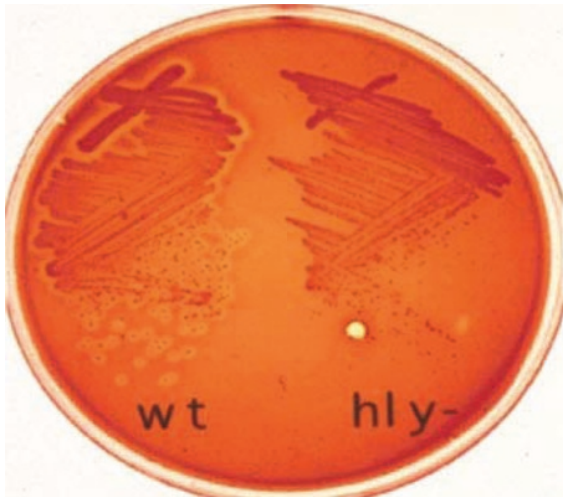


Fig. 8. Wild type (WT) *E. tarda* and a nonhemolytic mutant (*hly*<sup>-</sup>) of *E. tarda* on blood agar. (From Strauss et al., 1997, with permission)

together. The hemolysin is also regulated by the availability of iron (Janda and Abbott, 1993; Fig. 9).

Elevated levels of hemolysin are produced by *E. tarda* strains under iron-limited conditions and two putative ferric uptake regulator (Fur) binding sites are on the 5' upstream region of the activation/secretion protein gene *ethB* overlapping the promoter region and ribosome-binding site (Janda and Abbott, 1993; Hirono et al., 1997). Thus expression is upregulated under iron-limited conditions and downregulated in the presence of an excess of iron.

A heat-stable (60°C, 30 min; 100°C, 15 min) enterotoxin has been detected in 3 of 25 *E. tarda* strains employing the rabbit ligated ileal loop assay (Bockemühl et al., 1983). This enterotoxin did not cause Chinese hamster ovary (CHO) cell elongation. Conceivably, this enterotoxin could be operative in cases of secretory diarrhea associated with *E. tarda*. Also, *E. tarda* has recently been shown to induce plasma membrane ruffling in HEP-2 cells, a phenomenon that does not correlate with adhering bacteria (Phillips et al., 1998). The authors suggest that this may involve a distinct pathogenic mechanism in *E. tarda*.

A number of other potential virulence factors have been identified in *E. tarda* strains. These include hemagglutinins, dermatonecrotic substances, siderophores, and resistance to complement-mediated lysis (Ullah and Arai, 1983; Wong et al., 1989; Janda et al., 1991a). Their possible role in pathogenicity is speculative at present.

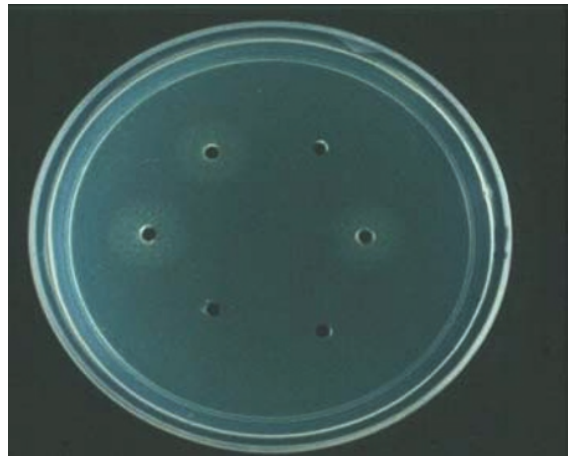


Fig. 9. Deferrated L agar seeded with *E. tarda* with sterilized suspensions of transferrin, hemin, ferritin, protoporphyrin IX, hemoglobin and hemein (clockwise, starting at 1 o'clock) added to individual wells. In this iron-starved medium, growth of *E. tarda* occurred only in the presence of hemin, hemoglobin and hemein.

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## The Genus *Citrobacter*

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### Introduction

Members of the genus *Citrobacter* are Gram-negative, nonsporeforming rods belonging to the family Enterobacteriaceae and, as the name suggests, usually utilize citrate as a sole carbon source. These facultative anaerobes typically are motile by means of peritrichous flagella. They ferment glucose and other carbohydrates with the production of acid and gas. They are oxidase negative, catalase and methyl red positive, Voges-Proskauer negative, and do not decarboxylate lysine. Taxonomically, the genus *Citrobacter* is most closely related to *Salmonella* and *Escherichia*.

In 1928, Braak described two strains of bacteria capable of forming trimethylene glycol from glycerol under anaerobic conditions. In honor of Freund's 1881 observation of trimethylene glycol as a fermentation product of glycerol, the strains were designated "*Bacterium freundii*" (Braak, 1928). One of these strains (ATCC 8454) was among the citrate-utilizing, lactose-fermenting coliforms that Werkman and Gillen designated "*Citrobacter freundii*" in 1932 (Werkman and Gillen, 1932). This classification was not universally accepted and several synonyms were used to describe these organisms including *Escherichia freundii*, *Colobactrum freundii*, *Paracolobactrum freundii*, *Salmonella ballerup*, *Salmonella hormaechei*, the Ballerup group, the Bethesda group, and the Bethesda-Ballerup group. In 1958, the International Subcommittee on the Taxonomy of the Enterobacteriaceae approved the name *C. freundii* for this heterogeneous group of bacteria. Subsequently, two groups of bacteria that were similar to *C. freundii* were recognized. The first was designated "*C. koseri*," "*C. diversus*," or "*Levinea malonatica*" by different researchers, and the other was designated "*L. amalonaticus*" (The Genus *Citrobacter* in the second edition). Eventually, these bacteria were included in the genus *Citrobacter*, and Frederiksen (1990) requested that the junior synonym *C. diversus* be formally replaced with *C. koseri*. In 1993, the Judicial Commission of the International Committee on Systematic Bacteri-

ology granted the request (Joint Commission of the International Committee on Systematic Bacteriology, 1993), and recognized *C. koseri* as the appropriate name. That same year, Brenner et al. (1993) introduced significant changes to the classification of the genus. Characterization of 112 strains for DNA relatedness using DNA-DNA hybridization resulted in the recognition of 11 distinct *Citrobacter* species. These groups were called "genomospecies" because the classification was based on DNA relatedness, but the species could also be distinguished by their biochemical profiles. In addition to *C. freundii*, *C. koseri* and *C. amalonaticus*, five new species (*C. farmeri*, *C. youngae*, *C. braakii*, *C. werkmanii* and *C. sedlakii*) were identified. The three other species (genomospecies 9, 10 and 11) were later named *C. rodentium* (Schauer et al., 1995), *C. gillenii* and *C. murliniae* (Brenner et al., 1999), respectively. A summary of the *Citrobacter* species and their type strains is presented in Table 1. More details on the historical taxonomy of the genus, up until the year 1992, can be found in the first and second editions of this text (Farmer, 1981; The Genus *Citrobacter* in the second edition).

Table 1. Species of the genus *Citrobacter* and their type strains.

Species <sup>a</sup>		Type strain	
Number	Name	ATCC number	GenBank accession
1	<i>C. freundii</i>	ATCC 8090	AF025365
2	<i>C. koseri</i>	ATCC 27028	AF025372
3	<i>C. amalonaticus</i>	ATCC 25407	AF025370
4	<i>C. farmeri</i>	ATCC 51112	AF025371
5	<i>C. youngae</i>	ATCC 29935	None
6	<i>C. braakii</i>	ATCC 51113	AF025368
7	<i>C. werkmanii</i>	ATCC 51114	AF025373
8	<i>C. sedlakii</i>	ATCC 51115	AF025364
9	<i>C. rodentium</i>	ATCC 51116	AF025363
10	<i>C. gillenii</i>	ATCC 51117	AF025367
11	<i>C. murliniae</i>	ATCC 51118	AF025369

<sup>a</sup>Original genomospecies numbers from Brenner et al. (1993); species names from Brenner et al. (1993), except *C. rodentium* (Schauer et al., 1995), and *C. gillenii* and *C. murliniae* (Brenner et al., 1999).

*Citrobacter* species are infrequently isolated from stool and occasionally cause infections in people and in animals. *Citrobacter freundii* and *C. koseri* are regarded as opportunistic pathogens, and most of the other species of *Citrobacter* have been isolated frequently enough from the urine, blood and wounds, that they are presumed to be pathogenic as well. Nonetheless, the clinical significance of individual species of *Citrobacter* as well as possible differences in pathogenesis remain largely undetermined. Representative biochemical reactions for the 11 *Citrobacter* species are presented in Table 2. A brief description of each species follows.

*Citrobacter freundii* is the type species of the genus. It produces H<sub>2</sub>S abundantly in triple sugar iron (TSI) agar or similar media (such as Kligler agar), but does not typically ferment sodium malonate or produce indole (Sedlak, 1973). Brenner et al. have subdivided the species into 7 biotypes (Brenner et al., 1999).

*Citrobacter koseri* does not produce H<sub>2</sub>S in TSI agar, but H<sub>2</sub>S is detected using more sensitive media such as ferrous chloride-gelatin or lead acetate. Lysine is not decarboxylated (Sedlak, 1973), and there is no growth in KCN (Brenner et al., 1993). *Citrobacter koseri* can be differentiated from other species of *Citrobacter* on the basis of its ability to produce acid from adonitol and D-arabitol.

*Citrobacter amalonaticus* is typically positive for indole production, negative for H<sub>2</sub>S production on TSI agar, and grows in KCN. It utilizes citrate but does not ferment malonate.

*Citrobacter farmeri* (Brenner et al., 1993) corresponds to species 4 and is named in honor of John J. Farmer III. It is positive for indole production and negative or delayed positive for citrate utilization. It produces arginine dehydrolase and ornithine decarboxylase; and produces acid from  $\alpha$ -methyl-D-glucoside, melibiose, raffinose, and sucrose; and can utilize benzoate, 4-hydroxybenzoate, malitol, D-melibiose, 1-*O*-methyl- $\alpha$ -galactoside, palatinose, protocatechuate, D-raffinose, and sucrose, but not *m*-coumarate, dulcitol, or malonate as sole carbon sources.

*Citrobacter youngae* (Brenner et al., 1993) corresponds to species 5 and is named in honor of Viola M. Young. It is typically indole negative, positive or delayed positive for citrate and arginine dihydrolase, and negative for ornithine decarboxylase. It produces acid from dulcitol but not from melibiose and is able to utilize dulcitol, 3-phenylpropionate, and L-sorbose, but not gentisate, 3-hydroxybenzoate, malonate, D-melibiose, 1-*O*-methyl- $\alpha$ -galactoside, 3-*O*-methyl-D-glucose, or tricarballylate as sole carbon sources.

*Citrobacter braakii* (Brenner et al., 1993) corresponds to species 6 and is named in honor of Hendrik R. Braak. It is characterized by variable indole production, positive or delayed positive citrate utilization, arginine dehydrolase, and ornithine decarboxylase activity. It does not produce acid from salicin or sucrose and can utilize *m*-coumarate, 1-*O*-methyl- $\alpha$ -galactoside, 3-phenylpropionate, and L-tyrosine (delayed), but not L-sorbose as sole carbon sources. Two biotypes have been described, which differ primarily in their ability to utilize 4-aminobutyrate, lactose and lactulose as sole carbon sources (Brenner et al., 1999).

*Citrobacter werkmanii* (Brenner et al., 1993) corresponds to species 7 and is named in honor of Chester H. Werkman. It is negative for indole production and ornithine decarboxylase, and positive for citrate and arginine dehydrolase. It does not produce acid from dulcitol, melibiose or sucrose, and utilizes *m*-coumarate, D-lyxose, malonate, 3-phenylpropionate, L-sorbose and D-tartrate, but not dulcitol, 4-hydroxybenzoate, D-melibiose or 1-*O*-methyl- $\alpha$ -galactoside as sole carbon sources.

*Citrobacter sedlakii* (Brenner et al., 1993) corresponds to species 8 and is named in honor of Jiri Sedlak. It is positive for indole production, arginine dehydrolase, ornithine decarboxylase, and positive or delayed positive for citrate. It produces acid from dulcitol and melibiose, but not from sucrose, and it utilizes benzoate, dulcitol, 4-hydroxybenzoate, *myo*-inositol, lactulose, malonate, 1-*O*-methyl- $\alpha$ -galactoside and protocatechuate, but not 5-ketogluconate or L-sorbose as sole carbon sources.

*Citrobacter rodentium* (Schauer et al., 1995) corresponds to *Citrobacter* species 9 and the species name refers to gnawing animals. It is negative for indole and H<sub>2</sub>S production, citrate utilization (within 2 days), growth in KCN, and arginine dihydrolase and is ornithine decarboxylase positive. It is typically nonmotile, but some reports describe delayed motility after 4 days (Brenner et al., 1999). *Citrobacter rodentium* does not produce acid from melibiose, sucrose, dulcitol or glycerol but is positive for malonate utilization. The organism has only been isolated from rodents and is the causative agent of transmissible murine colonic hyperplasia (Luperchio and Schauer, 2001). It is the only known attaching and effacing bacterial pathogen of mice.

*Citrobacter gillenii* (Brenner et al., 1999) corresponds to species 10 and is named in honor of George Francis Gillen. It is negative for the production of indole and ornithine decarboxylase, positive for utilization of malonate, delayed positive for growth on citrate, and usually delayed positive for the production of arginine dihydrolase. In addition, it is negative for urease

Table 2. Biochemical reactions of *Citrobacter* species.

Test <sup>a</sup>	<i>C. freundii</i>	<i>C. koseri</i>	<i>C. amalonaticus</i>	<i>C. youngae</i>	<i>C. braakii</i>	<i>C. werkmanii</i>	<i>C. sedlakii</i>	<i>C. farmeri</i>	<i>C. rodentium</i>	<i>C. gilleni</i>	<i>C. murinitiae</i>
Indole	12	94	94	10	21	0	100	100	0	0	100
Citrate	80 (90)	100	100	74 (90)	75 (88)	92	83 (100)	0 (36)	0 (67)	50 (100)	88 (100)
H <sub>2</sub> S	59 (63)	0	12	74 (85)	41 (45)	92	0	0	0	83	75
Urease	57 (73)	59 (71)	88 (94)	69	38 (58)	92	100	43	83 (100)	0	63 (75)
Arginine	51 (100)	94 (100)	76 (94)	54 (90)	54 (100)	77 (92)	100	100	0	17 (83)	50 (88)
Ornithine	12 (14)	100	94 (100)	3	88	0	100	100	100	0	0
Motility	88	94	88 (94)	97	79	100	100	100	0 (33)	83	100
KCN	90 (100)	0 (6)	100	92 (95)	96 (100)	100	100	100	0	100	100
Malonate	8	94	0	3 (5)	0	92	100	0	100	100	0
Acid from											
Lactose	86 (92)	71 (88)	50 (100)	18 (90)	81 (86)	23 (77)	100	4 (100)	100	67 (83)	63 (100)
Sucrose	73 (78)	47	6	21	13	0	0	100	0	17	25
Dulcitol	24	41	0	74	46	8	100	0	0	0	100
Salicin	6 (16)	6 (88)	18 (94)	5	0 (8)	0 (8)	17 (50)	0 (93)	0 (100)	17 (83)	13 (63)
Raffinose	73 (86)	0	0	8	17 (21)	0	0	100	0	17	13
Esculin	0 (10)	0 (29)	0 (35)	3	0 (4)	0 (8)	17 (50)	0 (36)	0 (83)	17 (50)	0 (100)
Melibiose	96 (98)	0	6	5	88 (100)	0	100	100	0	83	38 (75)
Glycerol	100	100	41 (47)	92 (100)	92	100	83	79	0 (83)	67 (83)	100
Nitrite	100	100	94	92	100	100	100	100	100	100	100
ONPG	88 (96)	100	94	95	83	100	100	100	100	83 (100)	100

Abbreviations: TSI, triple sugar iron; ONPG, and *o*-nitrophenyl- $\beta$ -D-galactopyranoside.

<sup>a</sup>Tests read after 2 and 7 days of incubation at  $36 \pm 1^\circ\text{C}$ . Results are expressed as percent of strains positive within 2 days, with the percent of strains giving delayed positive reactions (3–7 days) in parentheses. A more complete list of biochemical test results can be found in the original publication. Citrate, Simmons; H<sub>2</sub>S, TSI; arginine, arginine deaminase; ornithine, ornithine decarboxylase; nitrite, NO<sub>3</sub>  $\rightarrow$  O<sub>2</sub>; and ONPG, *o*-nitrophenyl- $\beta$ -D-galactopyranoside. Modified from Brenner et al. (1999).

production and dulcitol fermentation and is unable to utilize gentisate, 3-hydroxybenzoate, 3-*O*-methyl-D-glucose, L-sorbose, or tricarballylate as sole carbon sources.

*Citrobacter murlinae* (Brenner et al., 1999) corresponds to species 11 and is named in honor of Alma C. McWhorter-Murlin. It is positive or delayed positive for indole production and citrate utilization, usually is delayed positive for arginine dehydrolase, and is negative for ornithine decarboxylase. It produces acid from dulcitol and esculin (delayed), grows on sodium acetate (usually delayed) but does not utilize malonate. Dulcitol, D-lyxose, 1-*O*-methyl- $\alpha$ -galactoside (delayed), and L-tyrosine, but not malonate or protocatechuate, can be utilized as sole carbon sources.

## Habitat

Most of what is known about the natural history of *Citrobacter* isolates is due to their occurrence as opportunistic pathogens in humans. They are believed to be commensal inhabitants of the intestine of humans and animals and, most likely as a consequence of fecal shedding, are present in the environment and can be recovered from water, sewage and soil (Sedlak, 1973; Brenner et al., 1999). Clinical isolates of *Citrobacter* species from humans have been characterized to some extent, but with the exception of *C. rodentium*, much less is known about *Citrobacter* isolates from animals and from the environment. In humans, *Citrobacter* are associated with urinary tract infections, wound infections, pneumonia, abscesses, septicemia, meningitis, and endocarditis in adults, as well as septicemia, meningitis, and brain abscesses in neonates (Graham and Band, 1981; Shamir et al., 1990; Tang et al., 1994; Shih et al., 1996; Arens et al., 1997; Dyer et al., 1997; Doran, 1999; Lu et al., 1999; Tellez et al., 2000; Lin et al., 2001; The Genus *Citrobacter* in the second edition).

*Citrobacter* accounts for approximately 1% of all cases of septicemia (Drelichman and Band, 1985). *Citrobacter* septicemia often occurs in elderly patients, is often hospital acquired, and almost always occurs in patients with underlying disease (Shih et al., 1996). The most common initial sites of infection are the urinary tract, the gastrointestinal tract, and wounds. Intra-abdominal tumors and hepatobiliary stones, in particular, have been implicated as common predisposing risk factors for *Citrobacter* septicemia (Drelichman and Band, 1985; Shih et al., 1996). Patients with *Citrobacter* bacteremia are more likely to have polymicrobial sepsis than patients with *E. coli* bacteremia. While *Citrobacter* septicemia is fatal in up to 50% of patients, the sever-

ity of the underlying medical condition clearly contributes to the unfavorable outcome.

*Citrobacter koseri* is an important cause of septicemia and meningitis in neonates and young infants (Graham and Band, 1981; Doran, 1999). A high percentage of these patients develop brain abscesses. While most cases are sporadic with no obvious source of infection, nosocomial outbreaks in neonatal care units have been reported. The outbreaks were apparently caused by person-to-person spread rather than acquisition from an environmental point source. Despite improvements in diagnostic imaging techniques and antibiotic therapy, one-third of these patients die, and few recover without sequelae (for a review see Doran, 1999). It has been suggested that intracellular survival and replication of *C. koseri* within macrophages contributes to the development of brain abscesses (Townsend et al., 2003), although the details of this process have yet to be determined.

The significance of *Citrobacter* species in animals has not been firmly established. Fecal carriage and extraintestinal infections occur in mammals, birds and reptiles, including snakes, lizards and tortoises. *Citrobacter rodentium* has been isolated from laboratory mice and gerbils and causes transmissible murine colonic hyperplasia (for a review see Luperchio and Schauer, 2001). A locus of enterocytes effacement (LEE) pathogenicity island encoding a dedicated type III secretion system and its effectors is present in *C. rodentium* but not in other *Citrobacter* species. This LEE pathogenicity island is highly homologous to cognate pathogenicity islands in enteropathogenic *E. coli* and enterohemorrhagic *E. coli*, and *C. rodentium* infection in laboratory mice serves as an important model system for studying the pathogenesis of these gastrointestinal pathogens of humans. As reported by Luperchio et al. (2000), *C. rodentium* is the only naturally occurring attaching and effacing pathogen of rodents, and all of the isolates characterized to date appear to be clonal.

## Isolation

Although there have been few efforts to selectively isolate *Citrobacter* species, they grow well on media commonly used for isolation of other Enterobacteriaceae, and selective media originally developed for the isolation of *Salmonella* have been used to isolate *Citrobacter* species from clinical specimens. These include enrichment media, such as tetrathionate (Miller), selenite F (Leifson), tetrathionate brilliant green (Kauffmann), and GN (Hajna). Plating media used for isolating *Salmonella*, including MacConkey lactose, Endo, desoxycholate cit-

rate (Leifson), bismuth sulfite (Wilson and Blair), and brilliant green (Kauffmann), can also be used to isolate *Citrobacter* species. Optimal temperature for growth is 30–37°C, and after 24 hours of growth, colonies are 2–3 mm in diameter. On MacConkey lactose agar, colonies can be red, pink or colorless, depending on how rapidly the individual *Citrobacter* isolate ferments lactose. Delayed utilization of lactose produces a characteristic “fisheye” colony morphology, featuring a pink or red center surrounded by a clear margin after 24 hours of growth.

### Identification

The current taxonomic classification of *Citrobacter* species is based on DNA relatedness. Routine identification of *Citrobacter* isolates is accomplished by performing selected biochemical and carbon source utilization tests. The biochemical profiles of the 11 *Citrobacter* species are summarized in Table 2. More details on biochemical test results as well as carbon utilization results for determining biotypes have been published by Brenner et al. (Brenner et al., 1993; Brenner et al., 1999) and Schauer et al. (1995). On the basis of these reactions, a simple dichot-

omous key was developed by O’Hara et al. (1995) for presumptive identification (Fig. 1). These authors also evaluated commercial identification systems for their ability to accurately identify *Citrobacter* species. Most of the problems with misidentification will be resolved when the databases are updated to reflect the revised classification system of 11, rather than 3, valid species of *Citrobacter* (O’Hara et al., 1995).

### DNA-based Systems

DNA RELATEDNESS (Hydroxyapatite Method of DNA-DNA Hybridization). Standards for genomospecies, or species defined by DNA relatedness, have been proposed as at least 70% related DNA under conditions optimal for DNA reassociation and 5% or less divergence (unpaired bases) within related sequences (Wayne et al., 1987). Brenner et al. (1999) used the additional criterion for defining *Citrobacter* species that DNA relatedness remain above 60% in reactions carried out under stringent conditions (75°C; whereas optimal conditions were 60°C). A summary of their DNA hybridization results is presented in Table 3.

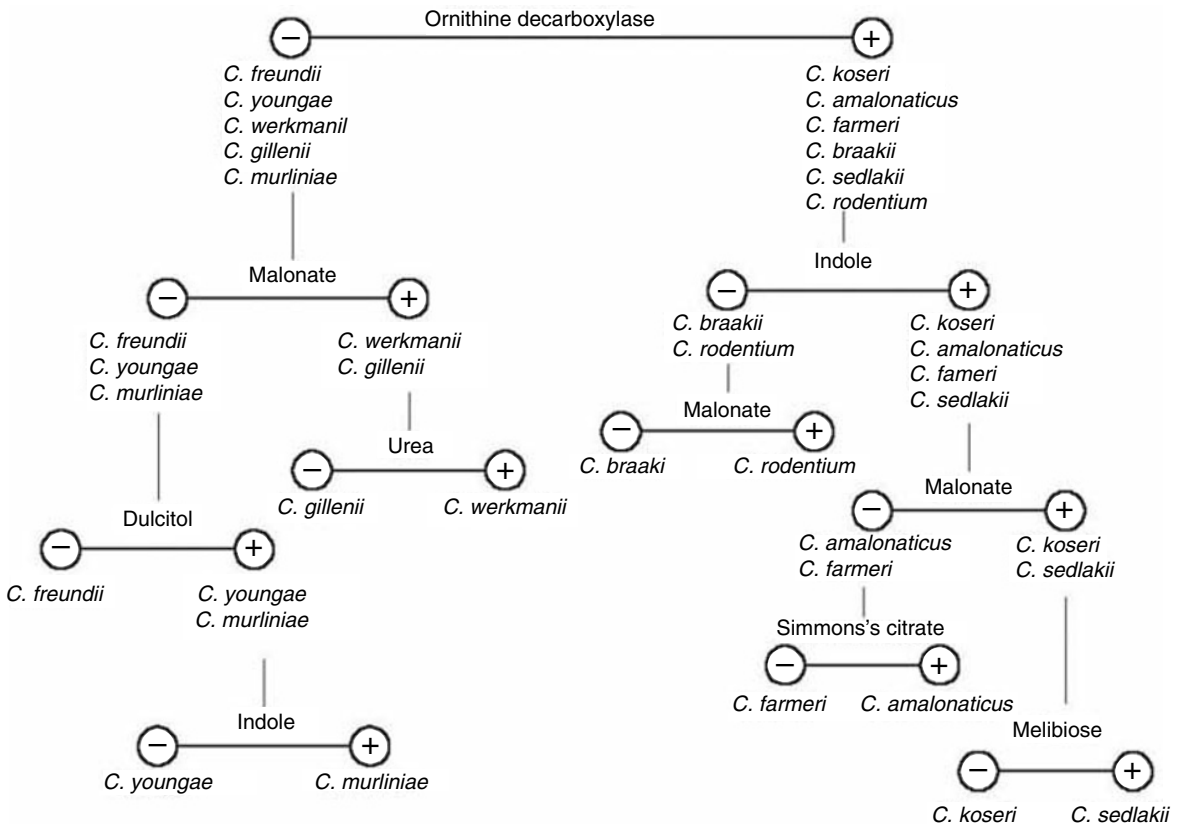


Fig. 1. Identification key for *Citrobacter* species, modified from O’Hara et al. (1995).



Table 3. DNA relatedness of *Citrobacter* species.<sup>a</sup>

Species	Number of strains tested	% Relatedness at 60°C	Divergence	% Relatedness at 75°C
<i>C. freundii</i>	46	85	1.5	79
<i>C. koseri</i>	2	79	0.5	75
<i>C. amalonaticus</i>	5	76	1.0	76
<i>C. farmeri</i>	1	71	1.5	70
<i>C. youngae</i>	29	81	2.5	77
<i>C. braakii</i>	16	82	0.5	79
<i>C. werkmanii</i>	10	78	2.0	77
<i>C. sedlakii</i>	6	83	1.0	78
<i>C. rodentium</i>	3	94	0.5	ND
<i>C. gillenii</i>	11	86	1.0	92
<i>C. murliniae</i>	7	91	0.5	91

Abbreviation: ND, not done.

<sup>a</sup>Modified from Brenner et al. (1999).

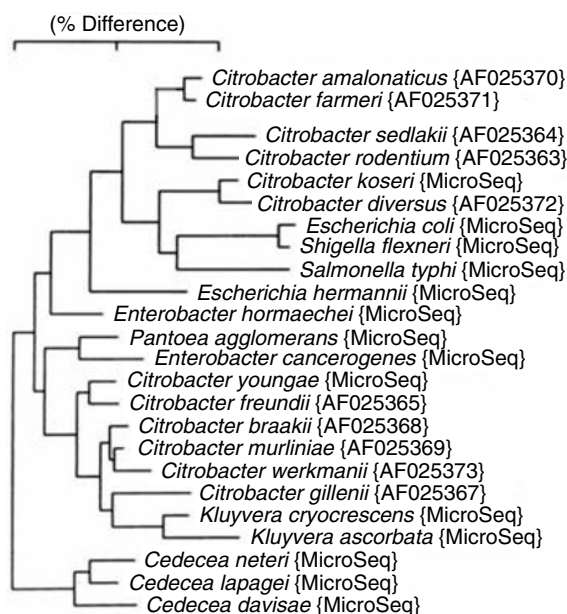


Fig. 2. Phylogenetic tree based on 16S rRNA sequence comparison, modified from Warren et al. (2000). The marker bar represents a 2% difference. GenBank accession numbers are listed. Microseq, sequences from the PE Applied Biosystems MicroSeq sequence database.

**SEQUENCE ANALYSIS OF THE SMALL rRNA SUBUNIT (PHYLOGENETIC ANALYSIS).** Genomespecies can also be defined on the basis of nucleotide sequence analysis of conserved portions of small rRNA subunits (Pace, 1997). Warren et al. (2000) have constructed a phylogenetic tree of the genus *Citrobacter* and related Enterobacteriaceae. The genus can be divided into three distinct groups, including 1) *C. freundii*, *C. youngae*, *C. braakii*, *C. werkmanii*, *C. gillenii* and *C. murliniae*; 2) *C. amalonaticus*, *C. farmeri*, *C. sedlakii* and *C. rodentium*; and 3) *C. koseri*, which is most closely related to *Escherichia coli*, *Shigella flexneri* and *Salmonella typhi* (Fig. 2).

**REPETITIVE EXTRAGENIC PALINDROMIC PCR (REP-PCR).** Phylogenetic relationships can also be deduced by means of high-resolution genotyping, such as repetitive extragenic palindromic polymerase chain reaction (rep-PCR) genomic fingerprinting. This method uses primers corresponding to naturally occurring interspersed repetitive elements in bacterial genomes (such as the REP, ERIC and BOX elements) for amplification of a series of products from genomic DNA (Versalovic et al., 1994). Rep-PCR profiles for the type strains of each *Citrobacter* species have been reported by Luperchio et al. (2000) and are presented in Fig. 3. Rep-PCR provides species-level identification for *C. rodentium*, with no apparent differences in amplification profiles between any of the isolates tested to date (Luperchio et al., 2000). However, for *C. koseri*, rep-PCR provides strain-level identification, with highly similar amplification profiles for epidemiologically linked isolates and distinct patterns for previously characterized strains within this species (Woods et al., 1992). The utility of rep-PCR for the other *Citrobacter* species has yet to be determined.

## Serology

A serotyping scheme for *Citrobacter* isolates was established by West and Edwards in 1954 featuring 32 O serogroups, 87 H serogroups, and 167 different O:H serotypes (West and Edwards, 1954). This system was extended by Sedlak and Slajsova (1966) to include 42–48 O serogroups and more than 90 H serogroups (for review, see Sedlak, 1973). Miki et al. (1996) identified the 90 reference strains of the serotyping scheme according to current species definitions. Among this group of strains (which included isolates of *C. youngae*, *C. braakii*, *C. werkmanii*, *C. gillenii*, but only a limited number of *C. freundii sensu stricto*), most serotypes were found to be species

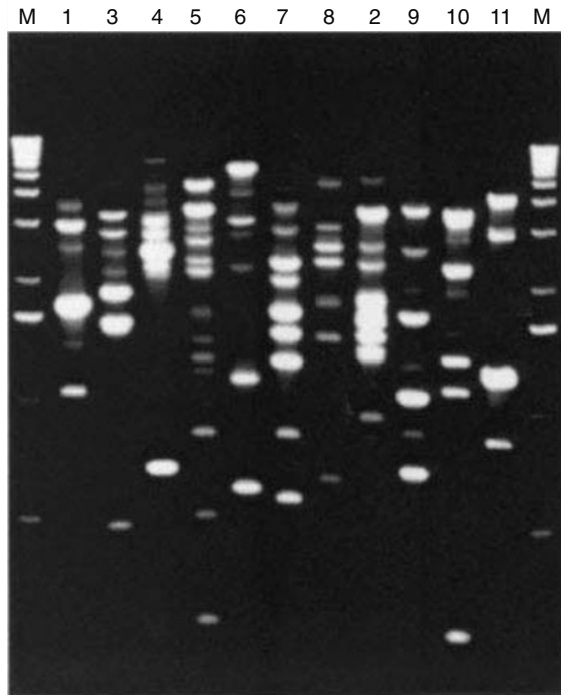


Fig. 3. Rep-PCR patterns amplified from DNA from *Citrobacter* species type strains, taken from Luperchio et al. (2000). Lane numbers represent the *Citrobacter* species numbers given in Table 1. M, 1-kb ladder standards.

specific. The serotyping system is not widely used for identification today, but it is important to recognize that serological cross-reactions between *Citrobacter* species and other Enterobacteriaceae (including *Salmonella* and *Escherichia*) do occur. Indeed, of the 20 chemotypes identified by Keleti and Luderitz (1971) based on the sugar composition of the lipopolysaccharide (LPS) of *Citrobacter* strains, 11 are identical to known *Salmonella* and *Escherichia* chemotypes. Cross-reactivity between *E. coli* O157 and *C. freundii* and *C. sedlakii* strains has been reported (Park et al., 1998; Nishiuchi et al., 2000; Vinogradov et al., 2000). Chemical structural data for the O-linked sugars of the LPS from a growing number of *Citrobacter* strains have been reported, including *C. freundii*, *C. youngae*, *C. braakii*, *C. sedlakii*, *C. rodentium* and *C. gillenii* (for a review, see Knirel et al., 2002).

## Antibiotic Susceptibility

Antibiotic resistance in *Citrobacter* species, as in other bacteria causing nosocomial infections, is an emerging problem. *Citrobacter* species without acquired antibiotic resistance are susceptible to sulfonamides, trimethoprim, aminoglycosides, chloramphenicol, tetracycline, nalidixic acid, flu-

oroquinolones, nitrofurantoin, polymyxins and fosfomycin (The Genus *Citrobacter* in the second edition; Pepperell et al. 2002). Like other Enterobacteriaceae, *Citrobacter* species are resistant to erythromycin and other macrolides, lincosamides, fusidic acid, and vancomycin (The Genus *Citrobacter* in the second edition). Acquired resistance to quinolones has been reported and is primarily attributable to mutations in *gyrA* encoding the A subunit of DNA gyrase, although enhanced efflux pump activity may also contribute (Navia et al., 1999; Tavio et al., 2000). Acquired resistance to aminoglycosides has also been reported and can be attributed to aminoglycoside acetyltransferase genes carried on plasmids or located in the chromosome, often in association with integrons (Hannecart-Pokorni et al., 1997; Wu et al., 1997).

Perhaps the most important aspect of antibiotic susceptibility in *Citrobacter* species concerns  $\beta$ -lactams. Even without acquired resistance, *Citrobacter* isolates are typically resistant to  $\beta$ -lactams such as ampicillin and carbenicillin. *Citrobacter* species that cluster with *C. freundii*, including *C. youngae*, *C. braakii*, *C. werkmanii* and *C. murliniae* (Fig. 2), possess an inducible class C  $\beta$ -lactamase on the chromosome that also confers resistance to early generation cephalosporins (Naas et al., 2002). *Citrobacter* species that cluster with *C. koseri*, including *C. sedlakii* and *C. rodentium* (Fig. 2), possess an inducible class A  $\beta$ -lactamase on the chromosome (Petrella et al., 2001). Antibiotic therapy with extended-spectrum cephalosporins is known to select for resistant *Citrobacter* isolates in vivo (The Genus *Citrobacter* in the second edition). In *Citrobacter* species with class C  $\beta$ -lactamase activity, resistance to extended-spectrum cephalosporins is associated with overproduction of  $\beta$ -lactamase due to mutations in the gene encoding AmpD (an amidase that interacts with AmpR, which is a transcriptional regulator of the LysR family). Resistance to extended-spectrum cephalosporins in *Citrobacter* species with class A  $\beta$ -lactamase activity is less well characterized.

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## The Genus *Shigella*

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### Introduction

*Shigella* is a Gram-negative, nonmotile, non-sporeforming, non-lactose-fermenting, facultatively anaerobic bacillus belonging to the family Enterobacteriaceae. It causes bacillary dysentery or shigellosis, as do enteroinvasive *Escherichia coli* strains (EIEC). The disease naturally occurs only in humans and primates. It is characterized, in its classical form, by acute colonic and rectal mucosal inflammation that leads to the triad of the dysenteric syndrome: fever, intestinal cramps, and passage of blood and mucus in diarrheal stools. *Shigella dysenteriae* 1 was the first of the four *Shigella* species to be described in 1898 by the Japanese microbiologist K. Shiga, thus the name of Shiga bacillus. The four species share several major characteristics such as lack of motility, optimal growth at 37°C, oxidase-negative character, and lack of growth in synthetic media containing salts and a simple carbon source, unless glucose and nicotinic acid are added. Some isolates may require the addition of certain amino acids, purines or vitamins. The chromosomal DNA of *Shigella* has a G+C content of about 50 mol%. The *Shigella* genus is very closely related to the species *Escherichia coli*; because the degree of homology of their chromosomal DNAs is close to 100%, considering *Shigella* as a member of the genus *Escherichia* is regarded as justifiable (Brenner et al., 1973). However, whereas *Escherichia coli* isolates are usually prototrophic, motile, and able to ferment many sugars, *Shigella* isolates are auxotrophic, nonmotile, ferment few sugars and, with a few exceptions, produce no gas in the presence of glucose. *Shigella* spp. therefore appear to be unique among the Enterobacteriaceae, in that their species status is maintained for reasons that are historical and linked to their particular metabolic profile and capacity to cause dysentery.

### Bacteriological Characters

The genus *Shigella* consists of four species, *S. dysenteriae* (subgroup A), *S. flexneri* (subgroup

B), *S. boydii* (subgroup C) and *S. sonnei* (subgroup D), characterized by biochemical and serological properties. The subgroup A differs from the other subgroups by its inability to ferment mannitol. *Shigella* serotypes are differentiated on the basis of their somatic O antigens.

*Shigella* spp. express numerous “negative” characters. Isolates are always nonmotile and nonflagellated. They do not grow on synthetic media with Simmons’ citrate, they do not express a deaminase for phenylalanine or tryptophan, and they are also negative for the following characters: urease, lysine and arginine decarboxylase, production of H<sub>2</sub>S (on triple-sugar-iron agar), growth in KCN medium, utilization of Christensen’s citrate or malonate, oxidation of gluconate, or liquefaction of gelatin. They give a positive methyl red and a negative Voges-Proskauer reaction. Like all *Enterobacteria*, *Shigella* spp. ferment glucose, but they (with the exception of *S. flexneri* 6, *S. boydii* 13 and 14 and *S. dysenteriae* 3) do not produce gas. Media containing lactose are not acidified by isolates belonging to *S. dysenteriae*, *S. flexneri* and *S. boydii* species. However, slow acidification (several days) may occur with *S. sonnei*. Adonitol, inositol and salicin are not fermented, but saccharose is slowly fermented by *S. sonnei*.

Mannitol is a key sugar in *Shigella* classification because, with rare exception, it is not fermented by *S. dysenteriae* and is fermented by all isolates belonging to the three other species.

The serotypic and biochemical characteristics that define the four species are summarized in Table 1 (Lindberg et al., 1991).

### *Shigella dysenteriae* (Subgroup A)

This subgroup comprises 12 serotypes, each possessing a distinct antigen. Crossreactions are very limited within this subgroup. Serotype 1 corresponds to the *Shiga bacillus*, which is also characterized by its production of the Shiga toxin (see below). Serotypes 2, 7 and 8 produce indole. *S. dysenteriae* 1 also produces a very active β-galactosidase (*o*-nitrophenyl-β-D-galactyl pyranoside [ONPG] test positive in less than 1 h) and

Table 1. Characteristics of *Shigella*.

	Mannitol	Indole	Serotypes	Antigens	Comments
<i>S. dysenteriae</i> (subgroup A)	–	–	1, 3, 4, 5, 6, 10, 11, 12, 13 2, 7, 8	Each serotype possesses a distinct antigen	ONPG+, catalase–, arabinose–
<i>S. flexneri</i> (subgroup B)	+	[–] [+] –	1, 2 3, 4, 5 6	Several antigenic determinants are shared by the species members. Coagglutination is possible and highly absorbed antisera are needed.	<i>S. flexneri</i> 6 var. Manchester and var. Newcastle are gas + <sup>a</sup>
<i>S. boydii</i> (subgroup C)	+	–  +	1, 2, 3, 4, 6, 8, 10, 12, 14, 18  5, 7, 9, 11, 13, 15, 16, 17	The 13 serotypes are antigenically distinct, excepted serotypes 10 and 11 sharing a common antigen other than their specific antigen.	<i>S. boydii</i> 10 is ADH+, arabinose+, threolose+ <i>S. boydii</i> 14 is gas+, mannitol–, maltose+, sorbitol–, arabinose+, threolose+, ADH+ <i>S. boydii</i> 17 is ADH–, arabinose+, threolose+ <i>S. boydii</i> 9 is ONPG+ (African strains may be lactose+)
<i>S. sonnei</i> (subgroup D)	+	–	Centains only one serotype.	It undergoes antigenic variation in phase I (smooth and virulent) and II (rough non-virulent). A plasmid encodes the form I antigen and the ability to invade intestinal epithelial cells. The phase I is not antigenically related to other <i>Shigella</i> groups but identical with <i>Plesiomonas shigelloides</i> serotype C27.	<i>S. sonnei</i> usually ferments lactose or saccharose after 3, 4 days at 37°C 4 major biotypes (99% of the strains): a: ONPG+, xylose–, rhamnose+ g: ONPG+, xylose–, rhamnose+ d: ONPG–, xylose+, rhamnose+ f: ONPG–, xylose–, rhamnose+

<sup>a</sup>Var Manchester is glucose +, mannitol +, and var Newcastle is glucose –, mannitol –.

does not produce catalase, an extremely unusual feature among Enterobacteriaceae. Certain isolates of *S. dysenteriae* 6 also may possess a  $\beta$ -galactosidase, but the ONPG test turns positive very slowly (1 day).

### *Shigella flexneri* (Subgroup B)

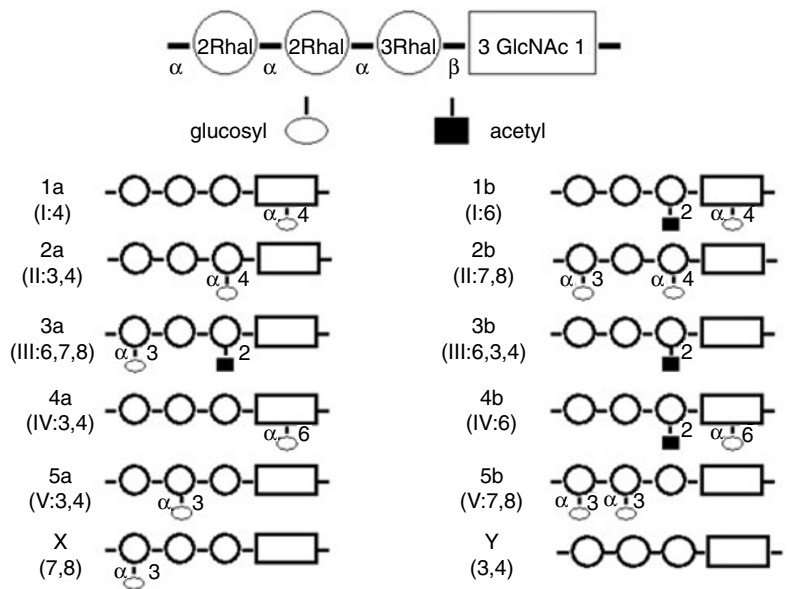
This subgroup comprises 6 serotypes whose antigenic schemes are summarized in Table 2. These six main serotypes are each characterized by a different specific type antigen. Serotypes can further be divided according to the group antigens. Subtyping requires highly absorbed antisera and can thus be performed only at reference centers where these antisera are available. Type antigens are designated by roman digits and group antigens by Arabic digits. With the exception of *S. flexneri* serotype 6 (whose assignment to subgroup B is still challenged by some authors), group 3,4 antigen is present as the primary structure (Simmons et al., 1987). Figure 1 shows the composition of the repetitive polysaccharide subunits (i.e., the somatic antigen) that polymerize as O-side chains of the lipopolysac-

Table 2. Serotypes of *Shigella flexneri*.

Serotypes	Subserotypes (group antigens)	Type antigens (antigenic formula)
1	1a	I:4
	1b	I:4,6
2	2a	II:3,4
	2b	II:7,8
3	3a	III:6,7,8,(3,4)
	3b	III:6,(3,4)
4	4a	IV:3,4
	4b	IV:6
5	5a	V:3,4
	5b	V:7,8
6	—	VI:(4)
X	—	—:7,8
Y	—	—:3,4

charide (LPS), supporting serotype specificity (Ewing, 1986). Some isolates have lost expression of their type-specific antigen, thus showing only their group antigen; they are identified as X and Y. Isolates of *S. flexneri* 1–5 may be indole positive.

Fig. 1. Sugar composition of repetitive subunits that compose the *S. flexneri* somatic antigens. Further glycosylation and/or acetylation defines serotype specificity.



### *S. boydii* (Subgroup C)

This subgroup has 18 serotypes, all of which are antigenically distinct, with two exceptions (serotypes 11 and 12), which share a common antigen that does not correspond to their specific antigen. Strains belonging to serotypes 5, 7, 9, 11, 13 and 15 are indole positive. Strains of serotype 14 are ADH positive and some isolates of serotypes 13 and 14 can produce gas in the presence of glucose.

### *S. sonnei* (Subgroup D)

Unlike other *Shigella* species, *S. sonnei* has a single serotype that undergoes a phase variation from a smooth, virulent, phase I to a rough, avirulent, phase II, which has lost the capacity to synthesize its O-side chains. The phase I antigen is not antigenically related to any other *Shigella* serotype, but is similar to serotype C27 of *Plesiomonas shigelloides*. The change from phase I to II is irreversible and has been shown to be due to the loss of a large virulence plasmid (Sansone et al., 1980; Sansone et al., 1982; Kopecko et al., 1980). Although corresponding to a single serotype, the species *S. sonnei* can be subdivided in biovars on the basis of the following characters: ONPG, xylose and rhamnose. The most frequent biovars are ONPG<sup>+</sup>, xylose<sup>-</sup>, rhamnose<sup>+</sup> and ONPG<sup>-</sup>, xylose<sup>-</sup>, rhamnose<sup>+</sup>, whereas the least frequent is ONPG<sup>-</sup>, xylose<sup>+</sup>, rhamnose<sup>+</sup>.

## Clinical Findings

The classical syndrome associated with *Shigella* is dysentery, which develops with an incubation

period of 1–4 days. However, the severity of dysentery varies widely from severe to asymptomatic infection (asymptomatic infections are common in highly endemic areas where up to 50% of all *Shigella* infections may be asymptomatic). In experimentally infected volunteers, the classical triad of shigellosis (fever, abdominal pain, and passage of blood and mucus in diarrheal stools) was observed only in half of those infected (DuPont et al., 1969). The disease usually begins with fever, anorexia, fatigue and malaise. Patients presenting with high fever, frequent bloody stools of small volume (sometimes grossly purulent), abdominal cramps or tenesmus, and showing large clumps of leukocytes in their feces, can be given presumptive diagnosis of bacillary dysentery. The typical illness usually begins with fever (38–40°C), with lower abdominal pain, watery diarrhea and malaise. Watery diarrhea may be brief or even absent. Severe dehydration is not a typical picture. However, in malnourished patients, the loss of water and electrolytes may cause dehydration and salt imbalance. Twelve to 36 hours later, diarrhea progresses to dysentery, blood, mucus and pus appearing in feces that decreases in volume (no more than 30 ml of fluid per kg per day). Stool frequency is variably increased, with 10–100 movements per day. During this second stage, shigellae multiply throughout the colonic mucosa, stimulating inflammatory infiltration with formation of abscesses. The pathogenesis of shigellosis involves invasion and inflammation of the colonic epithelium, destruction of the superficial mucosa, and production of ulcers. Recovery of bacteria in the blood is rare.

The disease always is localized to the rectosigmoid area, whereas more proximal portions of

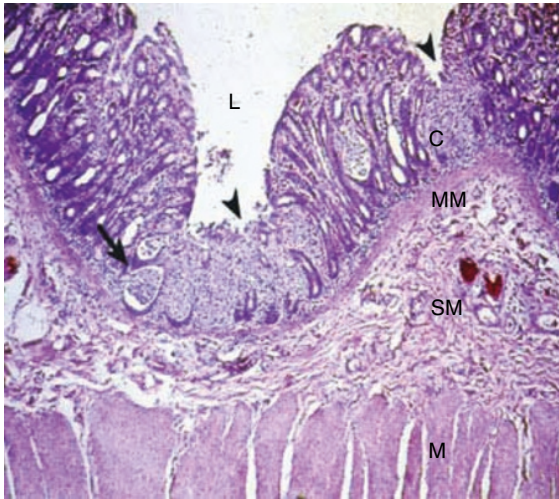


Fig. 2. Mucosal inflammation and abscess formation in colonic mucosa in the course of shigellosis. L = intestinal lumen, C = crypt, MM = muscularis mucosae, SM = submucosa, M = mucosal layer. Arrowhead points to an abscess ulcerated into the colonic lumen. Arrow points to a crypt abscess (i.e., cryptitis). Picture is a gift from S. B. Formal.

the large intestine are less frequently affected. While dilated colonic crypts are common during early stages of dysentery, elongated crypts are seen only when the duration of disease exceeds seven days on average. In about 30% of patients, abscesses develop because crypts become heavily infiltrated with polymorphonuclear leucocytes. Histological damage is mostly in the distal large bowel (Fig. 2). A proctoscopy examination during this phase shows a swollen mucosa with mucus, hemorrhages, and ulcers resulting from the death of colonic mucosal cells, detachment of the epithelial layer, and rupture of the abscesses. The lesions are continuous and diffuse and may involve edema, erythema, focal hemorrhages, and occasionally a white mucopurulent layer that adheres to the intestinal wall. Bacteria usually are confined to the epithelium of the surface and upper third of the colonic crypts. An exudate consisting of colonocytes, polymorphonuclear granulocytes with whole or partly digested bacteria, and erythrocytes in a fibrin mesh may be found in the intestinal lumen. Damaged epithelial cells are shed and goblet cells are reduced in number. The epithelial monolayer frequently is infiltrated by polymorphonuclear cells, lymphocytes, and mast cells. In the crypt epithelium, activated intraepithelial lymphocytes are prevalent, and monocytes arrive at a later stage of the dysentery. Ulcerations may extend into the lamina propria and associate with an inflammatory infiltrate in the submucosa. Widespread vascular lesions also are seen, ranging from swollen or pyknotic vascular endothe-

lial cells to complete destruction of the endothelium.

Progression to clinical dysentery occurs in most patients with *S. dysenteriae* type 1 and is also common in *S. boydii* and *S. flexneri* infection. Conversely, infections due to *S. sonnei* are, in general, less severe than those caused by other subgroups; they are often associated with vomiting and watery diarrhea, and possibly with dehydration.

In the poorest areas, persistent diarrhea and chronic malnutrition are the most common chronic complications (Black et al., 1982). This chronic malnutrition syndrome may last for weeks to months, thus affecting growth, owing to protein-losing enteropathy. If the negative nitrogen balance is not corrected during convalescence, protein loss may cause severe growth retardation.

Although *S. dysenteriae* type 1 is the *Shigella* species that is most often associated with severe bacillary dysentery and complications, all *Shigella* species may give rise to fatal disease. Septicemia is rare, but episodes of *Shigella* bacteremia have been reported during bacillary dysentery, especially among malnourished children (Struelens et al., 1985) or adult patients with impaired immunity. In immunocompetent patients, when bacteremia occurs, a non-*Shigella*, Gram-negative species is isolated in about half of the cases (Bennish et al., 1991). Other reported complications include hypoglycemia (which may be lethal in infants; Bennish et al., 1990a), seizures (Bennish, 1991a), as well as rectal prolapse, toxic megacolon, hemolytic-uremic syndrome (HUS), and leukemoid reaction (Butler et al., 1984). Toxic megacolon may lead to intestinal perforation and HUS may cause death by renal failure and/or anemia. Shiga toxin is implicated in HUS, but the mechanisms behind the pathogenesis of these complications remain incompletely understood (Karpman et al., 1997). Post-*Shigella* reactive arthritis or full-blown Reiter's syndrome (sterile arthritis, uveitis and urethritis) are classical complications, particularly in persons expressing the HLA-B27 histocompatibility antigen.

## Epidemiology

### General Considerations

*Shigella* generally is considered to have a narrow distribution in nature, inhabiting essentially the intestinal tract of humans, as well as captive primates in which shigellosis naturally occurs. There is no evidence, however, that the disease naturally occurs, particularly in the wild in those monkeys, without prior contact with humans



(Carpenter et al., 1965; Takasaka et al., 1964). Although shigellae are difficult, if not impossible, to grow from environmental samples, they are consistently present, particularly in sewage, and accidental contamination of water supplies by sewage effluents is regularly followed by an outbreak of shigellosis. Most cases of shigellosis are individual cases following person-to-person transmission. Outbreaks are usually a response to food and/or water contamination.

The disease is endemic throughout the world, although 99% of the cases occur in the developing world. Shigellosis is a disease that affects the poorest populations of the planet. According to a review by Kotloff et al. (1999), the total number of *Shigella* episodes that occur each year throughout the world is estimated to be 164.7 million, including 163.2 million cases in developing countries, 1.1 million of which result in death. Children under 5 years account for 69% of all episodes of shigellosis and 61% of all deaths attributable to shigellosis. Although distributed throughout the world, the prevalence of bacillary dysentery differs from place to place. It is estimated that the incidence of shigellosis (cases per 100,000 per year) is 6.5 in the United States, 130 in Israel, 3.3 in the United Kingdom, 5.6 in Australia, and 1.8 in France (Kotloff et al., 1999). Epidemics of shigellosis always have occurred among humans who gather in poor hygienic conditions: armies during military campaigns, pilgrimages, and refugee camps. In spite of the numerous epidemiological studies available, there is still a great need for accurate and frequently updated evaluation of the disease burden.

**TRANSMISSION** Transmission of *Shigella* occurs through the fecal-oral route. It is typically spread by direct contact with an infected person because the bacteria can survive on skin. Studies have demonstrated that an important vehicle for transmission of shigellosis is the hand, and that *S. dysenteriae* type 1 survives for up to one hour in culturable form on a human's skin (Islam et al., 1997). A characteristic of shigellosis is the ease with which person-to-person transmission of the pathogens occurs (Mosely et al., 1962). This is presumably due to the very small infectious inoculum required. Studies on American volunteers experimentally infected with *Shigella* have shown that as few as one hundred shigellae given orally cause the disease in 25–50% of the cases (DuPont et al., 1989). Resistance of *Shigella* to gastric juice certainly accounts, although not exclusively, for this high infectivity. *Shigella* also can be transmitted by contaminated foods (lettuce, onions, coconut milk desserts, hamburgers, raw oysters, meat, soup and pasta dishes), thus resulting in outbreaks (Wennerås et al.,

2000; Rennels et al., 1986; Boyce et al., 1982). Flies may transmit shigellae from human feces to foods (Hardy et al., 1948; Levine et al., 1991). In water, shigellae can survive for at least six months at room temperature. When outbreaks occur in a day care setting, attack rates may be as high as 33–73% (Weissman et al., 1974; Kotloff et al., 1999; Pickering et al., 1990), and secondary cases may be detected in 26–33% of the families of those children, thus confirming the high infectivity of these pathogens, as well as the significant role of day care centers in the dissemination of bacillary dysentery to the community (Kotloff et al., 1999; Pickering et al., 1981; Weissman et al., 1975). Oral/anal and oral/genital sexual practices also represent means of transmission (Dritz et al., 1974; Drusin et al., 1976) and may explain, in part, why shigellosis became so prevalent among the homosexual community in San Francisco in the early eighties (Tauxe et al., 1988; Laughton et al., 1988; Quinn et al., 1983).

### Shigellosis in Developing Countries

Shigellosis is associated mainly with conditions that characterize underdeveloped countries, such as malnutrition, poor personal hygiene, poverty, overcrowding, absence of food control, and inadequate water supplies. *Shigella* is a serious problem in developing countries because malnourished individuals suffer higher complication rates and prolonged disease. The disease may be fatal, especially among young children, as observed in Bangladesh (Bennish et al., 1991) where a *S. dysenteriae* type 1 epidemic was associated with a 42% increase in mortality in children aged 1–4 years. Since the late sixties, epidemics of bacillary dysentery involving *S. dysenteriae* type 1 have appeared in Central America (Gangarosa et al., 1969; Mata et al., 1969), sub-Saharan Africa (largely in refugee camps; Osisanya et al., 1988; Casalino et al., 1988), Central Africa (Ries et al., 1994; Germani et al., 1998a; Malengreau et al., 1983), South and Southeast Asia (Punyaratabandhu et al., 1991; Huilan et al., 1991; Adkins et al., 1987), and the Indian subcontinent including India (Rahaman et al., 1975), Bangladesh and Pakistan (Huilan et al., 1991). It also affects populations in areas of natural disaster (Kotloff et al., 1999; Keusch et al., 1989; Mosely et al., 1962). When multiresistant strains occur in these populations, or when a serotype is introduced that did not circulate before in the population, high attack rates are observed, and shigellosis often becomes a leading cause of death (Ries et al., 1994; Sack et al., 1997). In countries where shigellosis is endemic, it often exhibits seasonal peaks that can vary from one country to another. In Egypt and

in Bangladesh (Keusch et al., 1989), the transmission peak is in the hot dry season (mainly by use of contaminated water for consumption and decreased individual hygiene due to water scarcity); whereas in Guatemala (Mata et al., 1969), the peak is in the rainy season (owing to water-washed transmission during heavy rains).

The burden of shigellosis recently has been revisited (Kotloff et al., 1999), thus allowing emphasizing three major epidemiological characteristics of the disease. Shigellosis is essentially a disease of the developing world with 163.2 million cases/year leading to 1.1 million deaths, whereas only 1.5 million cases are observed in industrialized countries where lethal forms are rarely observed.

Shigellosis is primarily a pediatric disease, 69% of the total cases and 61% of the deaths occurring in children between 1 and 5 years old. In addition, severe diseases occur in non-breast-fed infants (Clemens et al., 1986) and shigellosis is uncommon in infants under 6 months (Wennerås et al., 2000; Kotloff et al., 1999; Bern et al., 1992). The rate of carriage may reach 1% among healthy children and 6% among children with other illnesses (Wennerås et al., 2000; Bennish et al., 1991; Hossain et al., 1994). Long-term carriage of *Shigella* (around one year) has been described in children (Wennerås et al., 2000). *Shigella* infections frequently are seen among children with chronic diarrhea, a condition that is frequently associated with malnutrition and stunted growth. Malnutrition is an important conditioning factor for lethal infection and not only predisposes for severe shigellosis, but the dysentery itself may give rise to negative caloric balance and growth retardation by several processes: protein loss during intestinal inflammation, food malabsorption, and lack of appetite. Often after resolution, catch-up growth does not occur (Bennish et al., 1991; Wennerås et al., 2000).

Unexpected mass displacement and gathering of people often face insufficient supplies of drinkable water, poor sanitation and malnutrition. In this setting, morbidity and mortality in all age groups can be important when outbreaks occur (Centers for Disease Control and Prevention, 1994). In 1994, during the epidemic of shigellosis in the former Zaïre, 20,000 patients died during the first month from bacillary dysentery among 800,000 Rwandan refugees. The causative strain was a multiresistant *S. dysenteriae* 1 isolate (Goma Epidemiology Group, 1995). High rates of morbidity and mortality also have been reported among refugees (Kotloff et al., 1999) from Somalia (Moore et al., 1993) and Kurdistan (Yip et al., 1993). *Shigella* is also a common etiologic agent of diarrhea among travelers to less developed regions, especially in the inter-tropical area. In their study, Kotloff et al. (1999) esti-

mated that the number of traveler's shigellosis cases originating from industrialized countries were 580,000 per year. Bacillary dysentery continues to affect soldiers during military campaigns (Felsen et al., 1945). Recently, during the Gulf War, 57% of the soldiers experienced an episode of diarrhea, and *Shigella* was isolated from 15% of the cases (Kotloff et al., 1999; Hyams et al., 1991).

Finally, increasingly in the developing world, an overlap is observed between zones of expanding HIV infection and endemic zones for shigellosis. Shigellosis in HIV-infected individuals shows more severe clinical patterns characterized by persistent or recurrent intestinal diseases as well as bacteremia (Angulo et al., 1995; Kristjansson et al., 1994).

### Shigellosis in Developed Countries

Bacillary dysentery in industrialized countries is prominently associated with disadvantaged populations. Persons that exhibit poor personal hygiene, such as those living in custodial institutions, or preschool children and toddlers in day care centers, can experience epidemics of bacillary dysentery or sustained endemic transmission (Tauxe et al., 1990). Although estimates in industrialized countries are based on cases reported to the national reference centers, these may underestimate the real incidence. While death is a rare outcome in developed countries, morbidity remains high when outbreaks occur in custodial institutions and day care centers (Wennerås et al., 2000; Kotloff et al., 1999). *Shigella sonnei* is frequently associated with cases occurring in young children in schools and day care centers in industrialized countries (Pickering et al., 1981; Thomas et al., 1973; Vagn-Hansen et al., 1991; Mohle-Boetani et al., 1995). Large urban outbreaks also have been described following accidental contamination of a city water supply by sewage effluents (Egoz et al., 1991).

### Distribution of Serogroups and Serotypes of *Shigella*

*Shigella flexneri* and *S. sonnei* account for the endemic form of shigellosis, whereas *S. dysenteriae* 1 accounts essentially for the epidemic form of the disease.

In developing countries, the most common *Shigella* species are *S. flexneri* (mainly serotypes 2a, 1b, 3a, 4a and 6) and *S. sonnei* (Kotloff et al., 1999). *Shigella dysenteriae* and *S. boydii* occur with equal frequency but *S. dysenteriae* type 1 is the most frequent cause of bacillary dysentery epidemics (Kotloff et al., 1999). *Shigella dysenteriae* 1 most often is identified in South Asia (Stoll et al., 1982; Khan et al., 1985) and sub-Saharan

Africa (Osisanya et al., 1988; Eko et al., 1991). It always has been distinguished by both its virulence and its ability to produce ravaging epidemics. *Shigella dysenteriae* 1 predominates in India (Stoll et al., 1982; Dutta et al., 1989), Malaysia (Jegathesan et al., 1984) and Guatemala (Ramiro Cruz et al., 1994), while type 2 predominates in Yemen (Al-Sallami et al., 1989) and Nigeria (Osisanya et al., 1988). *Shigella boydii* is uncommonly encountered except in the Indian subcontinent where it was first identified (Stoll et al., 1982; Dutta et al., 1989; Khan et al., 1985). In contrast, when the economy of a country improves, a switch occurs and epidemics usually are caused by *S. sonnei* (Kotloff et al., 1999). In the United States, the majority of bacillary dysentery cases are caused by *S. sonnei* infections. Among the *S. dysenteriae* isolated in the United States, types 1, 2 and 3 predominate. Serotype 2 predominates among *S. boydii* isolates (Bean et al., 1995). Limited data is available on serotypes isolated from travelers. In Finland, between 1985 and 1988, among 175 *Shigella* isolates from travelers, 75% were *S. sonnei*, 25% *S. flexneri*, 3% *S. boydii*, and less than 1% *S. dysenteriae* (Heikkila et al., 1990). Similarly, among 235 strains isolated from Japanese travelers, 64% were *S. sonnei*, 25% *S. flexneri*, 8% *S. boydii* and 3% *S. dysenteriae* (Ueda et al., 1996). Recently, in the course of Operation Restore Hope in Somalia, the serotyping of 37 *Shigella* isolates yielded 23% *S. sonnei*, 43% *S. flexneri*, 19% *S. boydii* and 15% *S. dysenteriae* (Sharp et al., 1995). Among *Shigella* strains serotyped during the Gulf War, 81% were *S. sonnei*, 11% *S. flexneri*, 7% *S. boydii* and 4% *S. dysenteriae* (Hyams et al., 1991).

## Pathogenicity

After oral inoculation, shigellae pass to the terminal ileum and colon where they invade and proliferate within epithelial cells, spreading from cell to cell. Bacillary dysentery is an invasive infection of the colonic and rectal mucosa that tends to remain local. In severe cases, the invasive process also may affect the terminal ileum. The disease process is characterized by an acute inflammation of the intestinal mucosa with ulcerations of the epithelium (Fig. 2). Why shigellae specifically invade the rectal and colonic mucosa is unknown. The bacteria rarely spread deeper in the lamina propria. Bacteremia occurs but is uncommon, except in malnourished children.

## Models of Infection

The pathogenesis of *Shigella* can be studied in various animal models that variably reproduce the invasive and proinflammatory capacity of the

microorganism. The almost exclusive specificity of *Shigella* for humans explains the lack of an animal model that mimics the natural disease. Nevertheless, several model systems manifest different facets of *Shigella* enteropathogenicity. The keratoconjunctivitis assay or Sereny test (Sereny, 1957) reflects the capacity of *Shigella* to penetrate into the epithelial cells of the conjunctiva and to spread from cell to cell, including corneal cells, thus making the cornea turbid, following massive inflammation caused by the dispersion of bacteria in tissues. The Sereny test remains the test of reference to assess the invasive capacity of a *Shigella* isolate in spite of its lack of relevance to invasion of the intestinal barrier. Similarly, a mouse respiratory infection assay, in which intranasally inoculated invasive shigellae cause acute invasive tracheo-bronchitis and condensing alveolitis (Voino-Yasenetsky et al., 1961), is now often used to detect pathogenicity. Three assays are more relevant to the invasion of the epithelial barrier: 1) the starved guinea-pig model (LaBrec et al., 1961), which is mainly a paradigm of mucosal translocation and animal death following systemic dissemination of shigellae and septic shock; 2) the rabbit ligated intestinal (ileal) loop assay, which manifests the rupture of the intestinal barrier, invasion and tissue destruction, and also allows study of the role of the follicle-associated epithelium that covers mucosa-associated lymph nodes in epithelial translocation of *Shigella* (Wassef et al., 1989; Sansonetti, et al., 1996); and 3) the oral or intragastric infection of macaque monkeys, which is the only model allowing development of true bacillary dysentery (Honjo et al., 1964). The latter's high price and low sensitivity however restrict its universal use.

Over the last years, major efforts have been devoted to the development of cell assay systems, mostly using epithelial cell lines (Nakamura, 1967; LaBrec et al., 1964) or macrophages. These systems, although restricted to demonstration of cell invasion (Fig. 3) and cell killing, have allowed identification of most of the genes that are relevant to expression of the invasive phenotype of *Shigella*.

## Natural History of the Disease

**PATHOGENESIS DETERMINANTS OF SHIGELLA**  
Virulent *Shigella* isolates express an invasive phenotype whose expression depends on the type of cell that is invaded by the pathogen (Fig. 4). Invasive shigellae enter epithelial cells by inducing a massive but local reorganization of the cell subcortical cytoskeleton, which results in a micropinocytic vacuole (Clerc et al., 1987) that engulfs the microorganism (Fig. 5). Internalized bacteria quickly escape the phagocytic vacuole

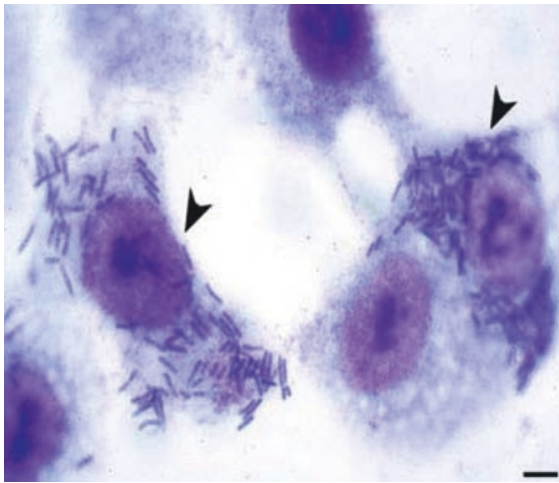


Fig. 3. In vitro assay: nonconfluent HeLa cells infected by an invasive strain of *S. flexneri*. Giemsa staining. Arrowhead points to intracellular bacteria.

#### EPITHELIAL CELL

Entry/Escape into cytoplasm  
Expression of proinflammatory potential (IL-8)



#### MONOCYTE/MACROPHAGE

Phagocytosis  
Apoptosis  
Expression of proinflammatory potential (Release of IL-1 $\beta$ )



#### POLYMORPHONUCLEAR LEUCOCYTE

Phagocytosis  
Release of granular content  
Increased adherence  
Killing of the bacteria



Fig. 4. Expression of the *Shigella*-invasive phenotype depending on the cell type under study.

and gain access to the cytoplasm in which they show rapid growth, as well as efficient actin-dependent intracellular motility and cell-to-cell spread (Makino et al., 1986; Bernardini et al., 1989). Simultaneously, the intracellular microorganisms reprogram infected epithelial cells to produce proinflammatory cytokines and chemokines (i.e., IL-8), essentially by inducing the translocation of NF- $\kappa$ B into the nucleus (Philpott et al., 2000), thereby transforming these cells into efficient mediators of the innate immune response. In addition, in the presence of

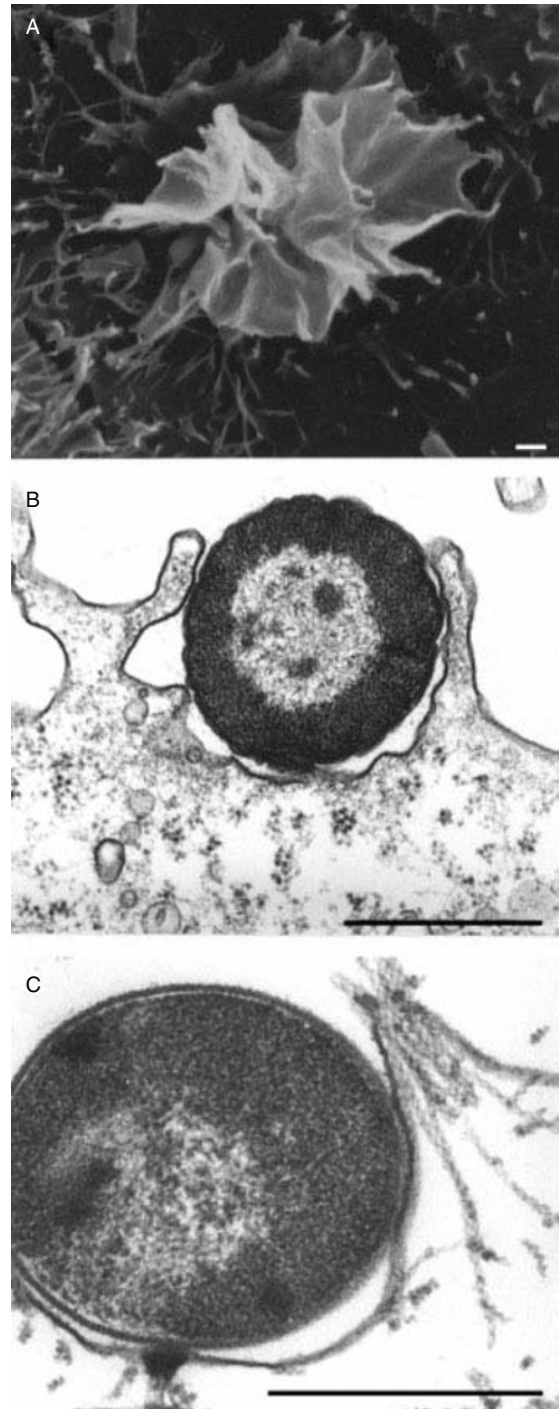


Fig. 5. In vitro assay: entry focus of *S. flexneri* into HeLa cells. A) Scanning electron micrograph showing the combination of filopodes and lamellipodes that make up the macropinocytic pocket that internalizes *Shigella*. B) Transmission electron micrograph showing section of a *Shigella* entry focus. Note the cellular extension that forms the macropinocytic vacuole. C) Transmission electron micrograph showing section of a *Shigella* entry focus. Actin filaments have been decorated with S1 myosin, thereby showing intense actin nucleation and polymerization in the submembrane area opposite to the bacterial body. Bar = 1  $\mu$ m. From the Station Centrale de Microscopie Electronique, Institut Pasteur.

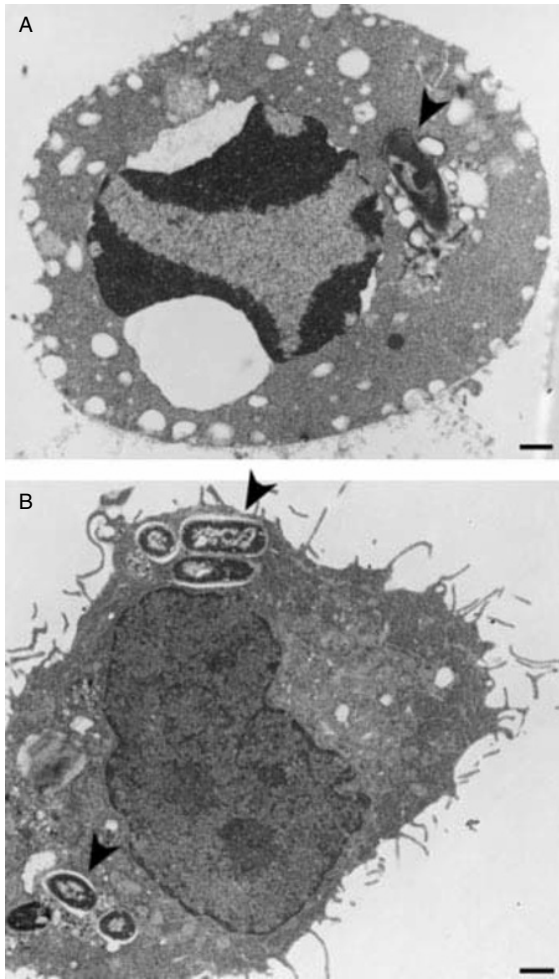


Fig. 6. In vitro assay: apoptotic death of J774 macrophages caused by invasive *S. flexneri*. A) Typical apoptotic macrophages observed after phagocytosis of an invasive strain of *S. flexneri*. Note peripheral condensation of chromatin in the nucleus and pit formation at the cytoplasmic membrane. B) Lack of macrophage apoptosis after phagocytosis of a non-invasive mutant of *S. flexneri*. Bar = 1  $\mu$ m. From the Station Centrale de Microscopie Electronique, Institut Pasteur.

lipopolysaccharide (LPS)-activated macrophages, invasive shigellae quickly cause apoptosis of these macrophages (Fig. 6) with concurrent release of massive quantities of the proinflammatory cytokine IL-1 $\beta$ ; Zychlinsky et al., 1992; Zychlinsky et al., 1994). Invasive shigellae also induce increased adherence and release of the granular content of polymorphonuclear leukocytes (PMNs), thereby further aggravating inflammation (Renesto et al., 1996), even though shigellae are eventually killed by these PMNs (Mandic-Mulec et al., 1997).

GENES ENCODING THE INVASIVE PHENOTYPE

All virulent *Shigella* organisms harbor 200- to 230-kb plasmids collectively named the “virulent plasmid” (Hale et al., 1983), which were first described for *S. flexneri* 2a (Kopecko et al., 1980) and *S. sonnei* (Sansone et al., 1980). These plasmids contain the genes required to express the invasive phenotype (Parsot et al., 1999). It was established that the loss of the virulence plasmid results in avirulent strains (Sansone et al., 1982) and that the genes implicated in the virulent functions are localized not only in the virulent plasmid but also in the chromosome (Hale, 1991). This plasmid is the “toolbox” that establishes a connection between the bacterium and its target cell. A key factor is a type III secretin (Fig. 7) that delivers *Shigella* effector proteins straight from the bacterial cytoplasm into the target cell membrane and cytoplasm (Blocker et al., 2000; Fig. 8). The *mxi* and *spa* operons encode proteins that assemble into a flagellum-like structure. Upon contact of the bacterium with its target cell, about 15 proteins are secreted that can be divided into two categories. The first category encompasses invasion-related plasmid-encoded antigens (Ipa)B, C and D, which are essential to the initial events of bacterial invasion of epithelial cells (Ménard

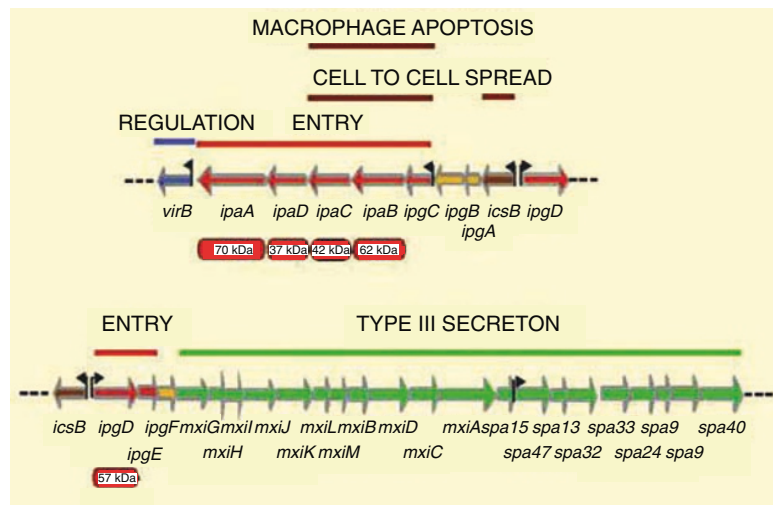


Fig. 7. The 30-kb pathogenicity island of *S. flexneri* carried by the 220-kb virulence plasmid. Currently known functions of the gene products are shown.

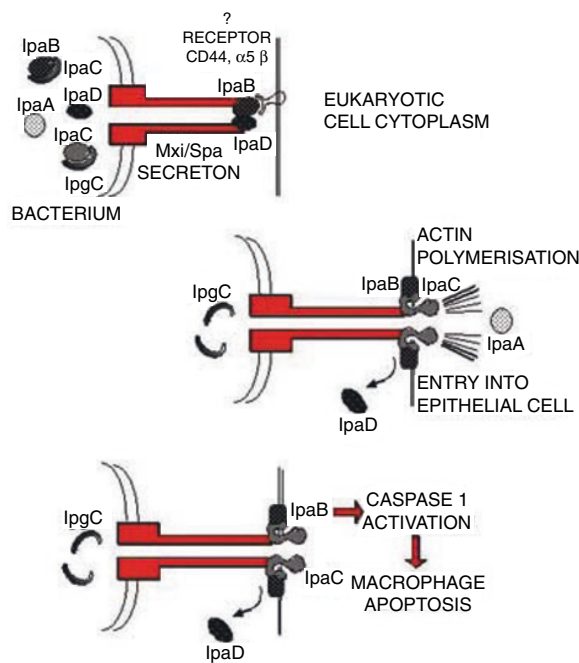


Fig. 8. Schematic description of *Shigella* interaction with epithelial cells and macrophages via its Type III secretin.

et al., 1993). A complex formed by IpaB and IpaD controls the flux of Ipa proteins through type III secretin (Ménard et al., 1994). Then IpaB and IpaC form a complex that inserts into the eukaryotic cell membrane, thus generating formation of a pore that serves two purposes. One is to channel proteins into the target cell cytoplasm; the other is to cause actin polymerization via IpaC (Tran Van Nhieu et al., 1999) and apoptosis of target macrophages via IpaB (Chen et al., 1996). The IpaC-mediated actin polymerization and formation of the *Shigella* entry-focus involves recruitment and activation of the small GTPases of the Rho family: Cdc42, Rac and Rho (Adam et al., 1996; Watarai et al., 1997; Mounier et al., 1999). This signalling cascade is regulated by tyrosine kinase and the protooncogene *c-src* (Dehio et al., 1995; Dumenil et al., 2000). A second set of proteins secreted through the type III secretin encompasses two subgroups: IpaA and IpgD, which are constitutively expressed at 37°C, regardless of the activation of the secretin (Demers et al., 1998). The IpaA protein binds and activates vinculin, a protein that is physiologically present in cell adherence plaques and activates the cell's capacity for bundling actin filaments, as well as an unexpected capacity to depolymerize actin. It thus appears that IpaA is essential to the maturation of the entry focus as well as its disappearance (Bourdet-Sicard et al., 1999). Similarly, IpgD seems to be essential to the maturation of the

*Shigella* entry focus. Once bacteria have been internalized, they quickly lyse their phagocytic vacuole and escape into the cytoplasm where they grow very efficiently. Transcription of the genes encoding the secreted, type III-dependent proteins other than Ipa proteins and IpgD is dependent on the activation of the type III system. This is the case for a multigenic family: the IpaH genes.

**OTHER *SHIGELLA*-PLASMID VIRULENCE-ASSOCIATED PROTEINS** The type III secretin and its target proteins represent the principal effector of *Shigella* interaction with both epithelial and phagocytic cells. However, other proteins are encoded by the virulence plasmid that are not targets of the type III secretin. Among these proteins, SepA is an IgA-protease-like serine protease that enhances inflammation of infected tissues (Benjelloun-Touimi et al., 1995). However, intra- or intercellular spread (IcsA) protein/VirG emerges as one of the major effectors of *Shigella* virulence (Makino et al., 1986; Bernardini et al., 1989). This 120-kDa outer-membrane protein of *Shigella*, which localizes at one pole of the bacterial body, mediates actin-based motility of the bacteria as well as their cell-to-cell passage. This protein recruits and binds N-WASP, a member of the Wiscott-Aldrich family of proteins. Binding of N-WASP on IcsA activates its binding function to a potent actin nucleator Arp2/3. This is sufficient to cause actin nucleation/polymerization at the bacterial surface, thus causing bacterial motility (Egile et al., 1999) as illustrated in Fig. 9. Actin-based motility, in the context of a confluent, polarized epithelium, allows cell-to-cell spread in a process involving the engagement of the components of the cell junction (such as cadherins and their associated molecules) by the bacteria (Sansonetti et al., 1994). A protrusion is formed that is engulfed by the adjacent cell. The two membranes that surround the bacterial body are then lysed, thus allowing escape of the bacterium into the cytoplasm of the adjacent cell. Expression of IpaB and IpaC is essential for completion of this lytic process (Page et al., 1999; Maurelli et al., 1998). This cycle of cell infection, which is summarized in Fig. 10, is superbly efficient in promoting bacterial intracellular colonization of the epithelium. Mutants in which *icsA* has been deleted are severely affected in virulence when tested in macaque monkeys (Sansonetti et al., 1991) as well as in human volunteers (Coster et al., 1999).

**CHROMOSOMAL VIRULENCE GENES** These genes can be classified in two major categories. The first is genes that regulate virulence gene expression on the plasmid, such as *virR*, a master regulator

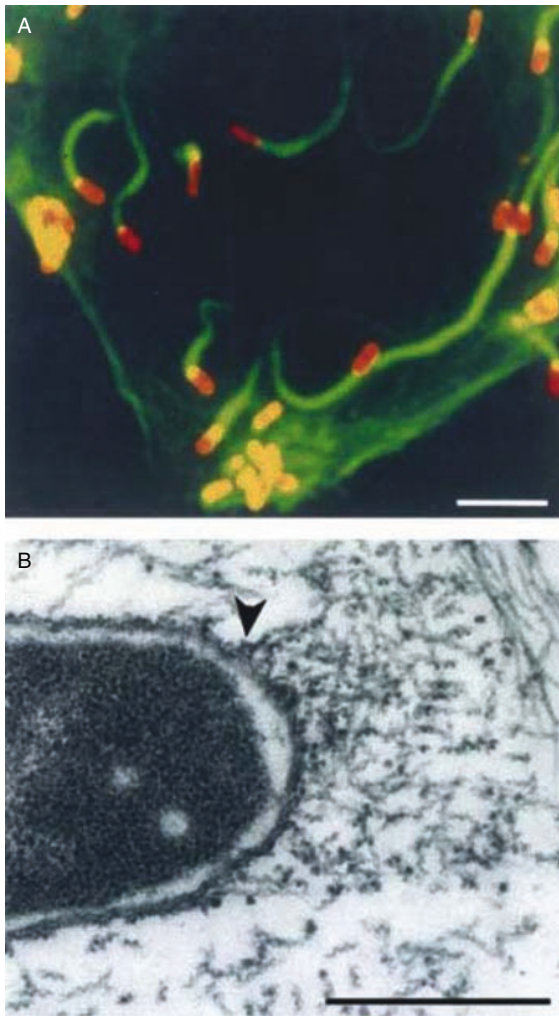


Fig. 9. In vitro assay: actin-dependent intracellular motility of *S. flexneri*. A) Actin comets trailing the moving bacteria. *Shigella* are stained in red with rhodamine-conjugated anti-LPS antiserum. Actin filaments are stained in green with FITC-phalloidin. Bar = 10 μm. B) Transmission electron micrograph of actin nucleation and polymerization at *Shigella* surface revealed by decoration with S1 myosin. Bar = 1 μm. From Station Centrale de Microscopie Electronique, Institut Pasteur.

that controls temperature-dependent expression of invasion genes (Maurelli et al., 1988) via its histone-like function (Dorman et al., 1990). The VirR protein directs a cascade of plasmid-encoded regulators such as VirF (which belongs to the AraC family; Sakai et al., 1986) and VirB (Tobe et al. 1991). The result is full expression of the invasive phenotype when bacteria are grown at 37°C and no invasion when bacteria are grown at 30°C. The second category comprises genes that are important for survival in the intestinal tract and in infected tissues such as those encoding the lipopolysaccharide and siderophores.

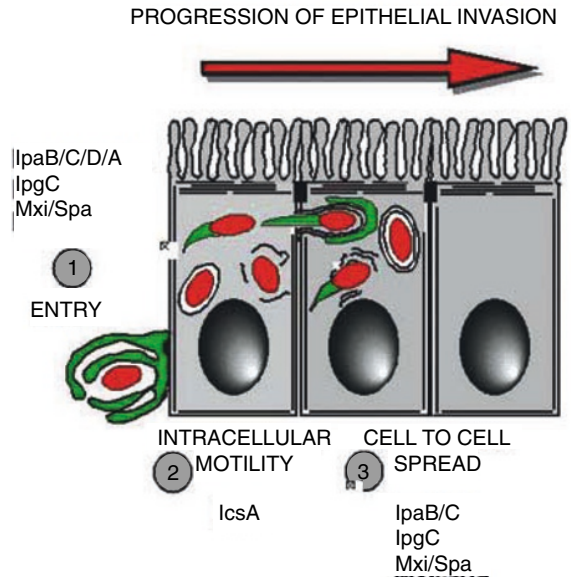


Fig. 10. Schematic representation of the major steps of epithelial invasion by *Shigella*.

Lipopolysaccharide. *Shigella* isolates that express a smooth LPS are virulent, whereas their rough mutants affected in expression of O-side chains are avirulent in in vivo assays such as the Sereny test. Also, LPS is the major constituent of the outer membrane. It has three covalently associated moieties: lipid A (responsible for the endotoxic activity of LPS), the core and O-side chains. The lipid A and core moieties are similar to their respective counterparts in *E. coli*. The O-side chains are composed of repeated sugar subunits that vary in their composition, thus accounting for the serotype diversity. In *S. flexneri* (Fig. 1), O-side chains are composed of a basic tetrasaccharide subunit constituting the serotype Y. Composition of this basic polysaccharide is 3β-N-acetyl-glucosamine-α1-2-rhamnose-α1-2-rhamnose-α1-3-rhamnose-1. Serotype variations, also called “serotype conversions,” are then conferred by temperate bacteriophages that carry glucosyl-transferases or acetyl-transferases that add glucosyl or O-acetyl groups to different sugars in the tetrasaccharide subunit (Allison et al., 2000). This provides the conversions leading to serotypes 1–5. *Shigella flexneri* serotype 6 remains an exception to this rule (Simmons et al., 1987). The principle of serotype variation in *S. flexneri* explains the frequent crossreactivity observed between those serotypes. Conversely, *S. sonnei*, whose genes encoding LPS O-side chains are located on the large virulence plasmid, presents a single—phase I—serotype, which is lost upon the loss of the virulence plasmid, thus leaving rough—phase II—clones (Sansonetti et al., 1980). In *S. dysen-*

*teriae* 1, the LPS-biosynthesis genes are located both on the chromosome and on a 9-kb plasmid (Watanabe et al., 1984).

When complementing an *E. coli* K<sub>12</sub> recipient by successive introduction of the *S. flexneri* virulence plasmid and chromosomal sequences from the same donor, expression of a smooth LPS appeared essential for transformants to express full virulence in *in vivo* assays such as the Sereny test and the rabbit ligated intestinal loop model (Sansonetti et al., 1983). Although rough strains are able to invade *in vitro*-cultured cells, they are impaired in their capacity to spread from cell to cell. This may be related to an indirect effect of the lack of O-side chains on the proper polar localization of IcsA, thereby altering actin-driven motility and cell-to-cell spread (Sandling et al., 1995). In addition, rough mutants of *Shigella* are hypersensitive to serum killing, and this sensitivity likely accounts for the lack of survival in inflamed tissues (Rowley, 1968).

**Iron Chelation.** Other virulence factors encoded by the chromosome of *Shigella* include siderophores and their surface receptors. Siderophores are low-molecular weight molecules with high affinity for ferric iron for which they compete against physiological carriers such as transferrin and lactoferrin. Most isolates of *S. flexneri* and *S. sonnei* express aerobactin siderophores, whereas, *S. dysenteriae* and some *S. sonnei* isolates express enterochelin receptors. Aerobactin-deficient mutants of *S. flexneri* are impaired in their capacity to grow in infected tissues (Nassif et al., 1987).

**Shiga Toxin.** In addition to the well-established virulence factors encoded by the chromosome in *S. dysenteriae* 1, Shiga toxin is encoded by a chromosomal gene designated “*stx*,” which maps near *pyrF*, at centisome 28 (Sekizaki et al., 1987; O’Brien et al., 1992). High levels of iron and reduced temperature repress production of Shiga toxin; iron regulation is governed by the ferric uptake regulator (Fur; O’Brien et al., 1987; Weinstein et al., 1988). Shiga toxin is a heterodimer that exerts potent cytotoxicity causing cell death by blocking eukaryotic protein biosynthesis. It shows a StxA(1)/StxB(5) stoichiometry. The StxA protein carries the catalytic activity, whereas StxB accounts for the binding of the toxin to Gb<sub>3</sub>, its ganglioside receptor molecule (Keusch and Jacewicz, 1977). Once internalized via receptor-mediated endocytosis in polarized epithelial cells, Shiga toxin reaches the Golgi apparatus from which StxA crosses the membrane and reaches the cytosol (Sandvig et al., 1991). A highly specific N-glycosidase, StxA removes adenine from one particular adenosine residue in the 28S RNA of the 60S ribosomal subunit of the eukaryotic cell target (Endo et al., 1988). This mode of action leads to arrest of pro-

tein biosynthesis, and cell death is strictly similar to that caused by plant toxins such as ricin and abrin (Saxena et al., 1989).

The function of Shiga toxin in eliciting diarrhea in the case of *S. dysenteriae* 1 infection has not yet been convincingly demonstrated in humans, although its enterotoxicity is clear in the rabbit ligated loop model (Butler et al., 1986). As the Gb<sub>3</sub> receptor for Shiga toxin is essentially detected in the non-epithelial fractions of intestinal tissues, it is possible that this toxin aims largely at non-epithelial targets in the intestinal mucosa and also may act systemically. Evidence indicates that Shiga toxin may primarily behave as a toxin for the vascular endothelium. Epidemiological data indicate that patients infected with *S. dysenteriae* 1 have more blood in their stools than patients infected with other species of *Shigella* (Stoll et al., 1982). In addition, macaque monkeys infected intragastrically with a wild-type isolate of *S. dysenteriae* 1 showed massive presence of blood in their stools, as well as major vascular destruction (particularly of capillaries of the lamina propria) in their intestinal tissues, compared to animals infected with a StxA-mutant, which showed less blood and limited vascular destruction (Fontaine et al., 1988). Some systemic manifestations also may be caused by Shiga toxin. This is particularly the case for the hemolytic uremic syndrome (HUS). Although the pathogenesis of this often fatal disease is not yet understood, its observation following infection with *S. dysenteriae* 1 and enterohemorrhagic strains of *E. coli* (EHEC) that produce Shiga-like toxins is a strong indication of a direct link. Histopathological analysis shows kidney lesions that are dominated by thrombosis and destruction of the glomerular capillaries, and sometimes cortical necrosis (Koster et al., 1978). The Gb<sub>3</sub> receptor is found in high density on human kidney endothelial cells, which are exquisitely sensitive to Shiga toxin (Obrig et al., 1993).

*Shigella* produces toxins other than Shiga toxin. Two enterotoxins have been described that may account for the early diarrheal phase often observed in the course of shigellosis. *Shigella* enterotoxin 1 is a chromosomally encoded toxin of 55 kDa that is essentially expressed by *S. flexneri* 2a (Noriega et al., 1995), and *Shigella* enterotoxin 2 is a plasmid-encoded enterotoxin of 63 kDa (Nataro et al., 1995).

Other potentially important components of virulence determination are chromosomal “black holes” (Maurelli et al., 1998), which correspond to genes that are deleted or not expressed, because their otherwise expressed product would alter virulence. This is the case for the *cad* gene encoding lysine-decarboxylase (LDC) function and the former *kcpA* gene



(keratoconjunctivitis production), which actually corresponds to the elimination of a lysogenic phage encoding a surface outer-membrane protein, OmpT. If expressed, this protease would cleave off the entire pool of surface-expressed IcsA, thereby leading to a phenotypically IcsA<sup>-</sup>, nonmotile, mutant (Formal et al., 1971).

**INTEGRATION OF PHENOTYPES** Shigellosis is prototypical of an infectious disease in which the pathogen manipulates innate immunity, particularly the inflammatory response, to disrupt and invade the intestinal mucosa. Severe intestinal tissue destruction may be the high cost that the host needs to pay to ensure bacterial eradication after a primary infection. Before eliciting this response, *Shigella* must cross the epithelial barrier, a process that can hardly be achieved through the apical pole of epithelial cells (Mounier et al., 1992; Perdomo et al., 1994a). Current evidence indicates that the initial entry route is through the follicle-associated epithelium (FAE) that overlays the lymphoid follicles associated with the intestinal mucosa, particularly those disseminated in the colonic and rectal mucosa (Sansone et al., 1999b). M-cells (essential functional components of the FAE) provide the initial route of bacterial translocation through the epithelium. These M-cells lack mucus and glycocalyx on their apical surface. They also are devoid of brush border microvilli. Consequently, this apical surface is easily accessible to pathogenic microorganisms of the intestinal lumen, and the high transcytotic activity of these cells facilitates translocation of the microbes through the epithelial lining straight into the inductive sites of mucosal immunity. M-cells form a pocket filled with both lymphocytes and resident macrophages (Neutra et al., 1996), thus providing direct contact between the intestinal flora and the mucosal-associated immune system (MALT). Selection of this route, which facilitates trans-epithelial translocation, however, directs the pathogenic microbe into an aggressive environment—the follicular dome—characterized by the density of resident macrophages. To survive, an invasive microorganism must evade the killing mechanisms of those phagocytic cells. Causing apoptotic death of its phagocytic macrophage seems to be the survival strategy that *Shigella* has selected (Zychlinsky et al., 1992; Zychlinsky et al., 1992). The secreted invasin IpaB of *Shigella* activates caspase 1 (a cysteine protease), which under these circumstances is able to induce the apoptotic cascade and proceed to rapid macrophage killing both in vitro and in vivo (Zychlinsky et al., 1996). Simultaneously, caspase 1 activation leads to the proteolytic cleavage of proIL-1 $\beta$ , thereby allowing for the release of mature IL-1 $\beta$  (Hilbi et al.,

1998), a potent proinflammatory cytokine that appears to be central in initiating mucosal inflammation in shigellosis (Sansone et al., 1995). This sequence of events causes a paradigm of “M-cell translocation—macrophage apoptosis—induction of inflammation,” which appears to be characteristic of the early stage of shigellosis. The concurrent decrease in IL-1RA, a non-agonistic competitor of IL-1 for its receptor, contributes to the inflammatory process (Sansone et al., 1999b). Simultaneously, caspase 1 activation also causes the proteolytic cleavage of IL-18, another proinflammatory cytokine, which is also a potent inducer of interferon  $\gamma$  (Fantuzzi et al., 1998), a cytokine that has been shown to be essential for the control of *Shigella* infection (Way et al., 1998). This concurrent event seems to be essential for the programming of the host antibacterial effect (Sansone et al., 2000). This is an example of the subtle balance that controls an infectious process. How could this benefit the microbe? It is actually paradoxical that such response does not lead to the immediate eradication of the invading pathogen. A combination of in vitro and in vivo experiments (Perdomo et al., 1994a; Perdomo et al., 1994b) have established another paradigm, also called “fatal attraction” in which the early inflammation serves as a “Trojan horse” for invasive shigellae. Inflammation that can be aggravated by a trans-epithelial signalling process disrupts the permeability of the epithelial barrier via induced transmigration of inflammatory cells, particularly polymorphonuclear leukocytes (PMNs), thereby allowing access of bacteria to the basolateral pole of epithelial cells, which is permissive to bacterial entry. Here, the invasive phenotype of *Shigella* takes full significance because penetration of bacteria into epithelial cells, as well as cell-to-cell spread, allows protection and diffusion of the microbe. As shown in Fig. 11, one can establish a scheme in which early entry at follicular sites followed by inflammation leads to centrifugal progression via the combined effect of inflammatory disruption of the epithelial barrier and epithelial cell invasion and cell-to-cell spread. In addition, IL-8 produced by infected epithelial cells causes massive attraction of PMNs that, at the cost of epithelial destruction, actively participate in the protection against bacterial translocation, mucosal diffusion, and systemic dissemination (Sansone et al., 1999b).

## Microbial Diagnosis

The diagnosis of shigellosis depends mainly on microbiological examination. For epidemiological studies, immunological assays for antibodies to *Shigella* LPS represent an alternative method

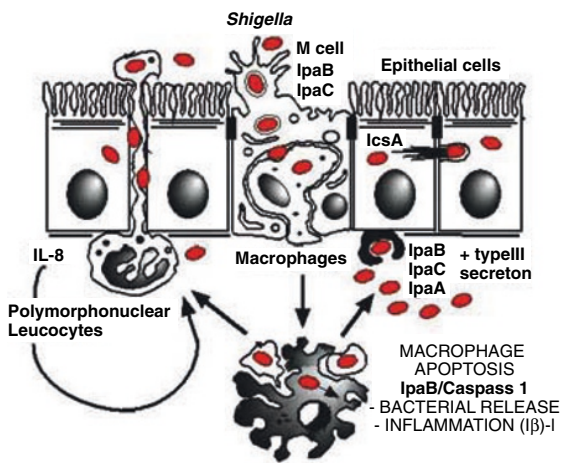


Fig. 11. Schematic representation of the crossing, rupture, invasion and inflammatory destruction of the intestinal barrier by *Shigella*.

to diagnose *Shigella* infection in retrospect (Lindberg et al., 1991; Cam et al., 1993). Bacteriological methods for isolation of *Shigella* from feces are widely available. *Shigella* are present in the feces of patients in concentrations ranging from  $10^3$ – $10^9$  viable bacteria per gram during the acute phase of the disease, but they readily die off if the stool sample is not well processed. *Shigella* may not survive changes in pH (acidification) owing to the growth of other bacteria or to a decrease of the temperature. *Shigella* are more fastidious and difficult to cultivate at later stages of the illness.

### Specimen Collection and Transport Media

To increase chances of successful isolation of *Shigella*, the specimens of choice are (in order of decreasing productivity) rectal swab specimens, fresh stools, and anal swabs collected during the active stage of the disease (Echeverria et al., 1991) before any antibiotic has been taken. Streaks of blood, mucus and pus, which are characteristic of dysenteric stools, should be cultured as they are likely to contain a dense population of almost pure *Shigella*. Once collected, these specimens should ideally be immediately inoculated onto culture media, as *Shigella* may not survive changes of pH resulting from overgrowth of other bacteria, or from a drop in temperature. If a delay in transporting stool or swab specimens is expected, direct inoculation into buffered glycerol saline (BGS), with an indicator to control the pH during transportation, is essential. Refrigerated BGS preserves *Shigella* better than the Cary-Blair transport medium. After inoculation, this transport medium should be used for plating on isolation media as quickly as possible (within 6 h).

### Isolation of *Shigella*

There is no effective and reliable enrichment procedure to ensure accurate determination of the presence of *Shigella* in feces, food or environmental samples. If the collection and handling of the specimens have been properly done, direct plating is therefore enough for successful isolation. Ideally, several media should be used to increase chances of isolation: one medium of low selectivity (i.e., MacConkey or Eosin-Methylene Blue [EMB] agar), one medium of high selectivity (i.e., Hektoen, *Salmonella-Shigella* [SS] or Xylose-Lysine-Deoxycholate [XLD] agar), and one nonselective medium (Bromocresol-Purple agar Lactose [BCP]). The choice of an optimal medium for primary culture of stool samples depends on the local conditions of work, on the prevalent strains in a region, and on the microbiologist's own experience. When working in the field, SS medium has the advantage of not requiring autoclaving, but is not adapted for *S. dysenteriae* type 1 and *S. sonnei* whose isolation is facilitated by the use of tergitol-7-tetrazolium agar. MacConkey and BCP agar plates appear to be the best media.

To optimize chances of recovering the very low numbers of *Shigella* found in some feces specimens, a portion of stool specimen may be inoculated into an enteric enrichment broth, such as Gram-negative broth, before plating onto the agar medium.

### Identification of *Shigella*

*Shigella* are non-lactose-fermenting enterobacteria. After 12–15 h of incubation of agar plates at 37°C, positive colonies that appear in positive cases will be selected for further examination. Such colonies may be subcultured on a more selective medium before complete identification. On MacConkey, Hektoen, BCP and SS agar, colonies appear translucent, and convex, with a smooth surface and a diameter between 0.5 and 1 mm. Suspect colonies can be identified directly in many commercial identification systems. In routine work, these colonies can be screened for *Shigella* genus by subculturing onto differential media to be distinguished from the other genera of the family Enterobacteriaceae. On Kligler-Iron-Agar (KIA), isolates that are glucose positive (acid yellow butt), lactose negative (alkaline red slant), H<sub>2</sub>S negative, gas negative, and non-motile on Motility-Indole-Urea (MIU) agar, can be further identified to genus and species. The four species of *Shigella* can be differentiated by biochemical properties (Table 1). *Shigella* are typically non-gas producing, with the exception of *S. flexneri* 6, *S. boydii* 13 and 14, and *S. dysenteriae* 3, all of which produce gas from glucose.

The four species are lysine decarboxylase<sup>-</sup>, acetate<sup>-</sup>, mucate<sup>-</sup>. Only *S. sonnei* ferments lactose slowly and ferments mucate, and some isolates can decarboxylate ornithine. Strains of *S. dysenteriae* are always negative for mannitol fermentation, are also catalase negative and occasionally *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) positive. *Shigella* species appear biochemically very similar, and differentiation among species depends on serological methods. In practice, serological methods using group- and type-specific antisera must be performed only after biochemical presumptive identification of *Shigella* species.

One can propose the following simple scheme for biochemical identification of *Shigella*:

Isolates are nonmotile and LDC negative;

With the few above-mentioned exceptions, isolates do not produce gas during carbohydrate fermentation.

Isolates fermenting mucate, or those alkaline on acetate or on Christensen's citrate agar, are likely to be *E. coli*.

Following this simple scheme, sero-agglutination should be performed.

### Serogrouping of *Shigella* Isolates

Although *Shigella* species can be identified presumptively at the genus level with biochemical tests as described above, their identity must be confirmed in slide agglutination with polyvalent (serogroups A–D) antisera. Agglutinating isolates suspected to be *Shigella* would then be tested by slide agglutination with monovalent, O-specific (serogrouping) antisera to identify the serogroup of a *Shigella* species. Below the species level, serotyping is usually done in reference laboratories. *Shigella* serotyping occasionally is affected by the transition from the smooth (S) form to the rough (R) form, and several intra- and interspecific crossreactions have been reported to interfere with the serotyping assay. The identification of *Shigella* serotypes by restriction of amplified O-antigen gene cluster was developed by Coimbra et al. (1999).

### Differentiation of *Shigella* spp. and *Escherichia coli*

*Shigella* spp. closely resemble *Escherichia coli* at the genetic level, and the biochemical identification sometimes requires additional tests (Table 1). Non-lactose-fermenting or anaerogenic strains of *E. coli* can be differentiated from *Shigella* by the following biochemical tests: Christensen's citrate, fermentation of sodium acetate and mucate, and decarboxylation of lysine. *Shigella* spp. are negative for these tests. When biochemically identified isolates of *Shigella* do not

agglutinate in *Shigella* antisera, a bacterial suspension should be heated (100°C for 15 min) and tested again for agglutination. In case of persistently negative agglutination, such isolates could correspond to enteroinvasive *E. coli* (EIEC) and should be sent to a reference laboratory for testing. The EIEC closely resemble *Shigella*; they are often lactose negative, lysine decarboxylase negative, and nonmotile (except for the O124 serogroup).

### Preservation of Cultures

Cultures of *Shigella* can be preserved for several years by conservation in Dorset egg agar or in cooked meat medium at room temperature. They also can be maintained either lyophilized or frozen at -80°C or in liquid nitrogen for very long periods. However, whatever conservation technique is selected, avirulent variants often arise owing to the loss or deletion of large fragments of the virulence plasmid. When reisolating a strain from a stock, care must therefore be taken to avoid reisolating and subculturing an avirulent (i.e., noninvasive) clone. Addition of Congo red to the agar medium allows identification of potentially invasive colonies (i.e., Congo red positive) that appear small, smooth, convex, and with a red central area of Congo red accumulation (i.e., rabbit-eye-like colonies), whereas noninvasive colonies (i.e., Congo red negative) appear large, flat and white, owing to an inability to accumulate the Congo red. In case of persisting doubt, colonies should be tested in an in vitro assay of invasion (i.e., invasion of cultured eukaryotic cells) or in the Sereny test.

### Molecular Diagnosis

Several key determinants involved in the pathogenicity of *Shigella* species and EIEC are plasmid- and chromosome-mediated. Investigators have developed DNA probes to diagnose and differentiate *Shigella* and EIEC from other non-pathogenic stool flora (review by Tenover et al., 1989).

Polymerase chain reaction (PCR) techniques applied to diarrheal stools (Luscher et al., 1994; Frankel et al., 1990; Achi et al., 1996) and food (Bef et al., 1991; Lindqvist et al., 1999; Villalobo et al., 1998) may be considered a more sensitive and specific technique than the conventional culture technique. The use of PCR to amplify a specific *virA* gene fragment (Villalobo et al., 1998) or the invasion plasmid antigen H (*ipaH*) DNA sequence (a multiple copy sequence found on the chromosome and the invasion plasmid; Sethabutr et al., 1993; Gaudio et al., 1997) serves as a specific and sensitive method to detect virulent bacteria of the genus *Shigella* and EIEC, and

has the potential to be employed in routine diagnosis of dysentery in clinical centers (Islam et al., 1998). Several methods, such as nested PCR amplifying sequences with an invasion-associated locus (*ial*; Lindqvist et al., 1999), multiplex PCR assay (Villalobo et al., 1998; Oyoyo et al., 1996), immunomagnetic separation-PCR assay (Achi et al., 1996; Islam et al., 1992), and automated methods of identifying PCR products (PCR-ELISA; Sethabutr et al., 2000) that process large numbers of specimens, facilitate epidemiological studies. A simple PCR procedure using IS 630-specific primers was developed as a general diagnostic probe to detect *Shigella* and EIEC (Houng et al., 1997). However, IS 630, *ipaH* and *ial* cannot be used to differentiate among *Shigella* serotypes and EIEC that cause dysentery. Serotype-specific primers derived from the *rfc* genes can differentiate among *Shigella* serotypes (such as *S. sonnei*, *S. flexneri* and *S. dysenteriae* 1) in the laboratory, and the multiplex PCR system containing *rfc*-specific primers can efficiently identify the most prominent *Shigella* serotypes in raw stool samples of diarrheal patients (Houng et al., 1997).

## Treatment of and Antibiotic Resistance to Shigellosis

Oral rehydration therapy provides little benefit to patients with dysentery and anti-diarrheal drugs, which decrease intestinal motility, may worsen bacillary dysentery, and should not be used in those circumstances (Wennerås et al., 2000; DuPont et al., 1973). In addition, the successful implementation of oral rehydration therapy in the developing world has increased the relative importance of dysentery and persistent diarrhea as a cause of mortality and morbidity in children (Khan et al., 1985). In a study carried out in Bangladesh, deaths attributed to dysentery among children 1–4 years outnumbered deaths attributed to watery diarrhea by a factor ranging between 2.1 and 7.8 (Bennish et al., 1991).

Antibiotics, on the other hand, shorten the duration of dysentery and bacterial excretion in stools (Haltalin et al., 1972). Although most cases of shigellosis are self-limiting and clinically benign, thus raising some controversy about the need to provide systematic antibiotic treatment, antimicrobial treatment is usually prescribed, particularly in severe cases. Unfortunately, over the years, multiresistant *Shigella* strains have become very common (Sack et al., 1997). *Shigella* quickly acquired plasmid-encoded resistance to the antimicrobial drugs that constituted first-line therapy. Throughout the world, *Shigella* isolates now express resistance to sulfonamides, trimethoprim-sulfonamides, tetracycline, ampicillin, chloramphenicol and streptomycin.

The *S. dysenteriae* type 1 strain that was responsible for the devastating outbreak in the former Zaïre among Rwandan refugees was resistant to all the commonly used antibiotics (Goma Epidemiology Group, 1995). In industrialized countries, multiresistant *Shigella* infections can be treated with a third-generation cephalosporin or a new 4-fluoroquinolone (Bennish et al., 1990b), but few reliable options exist in developing countries where the cost and practicality are paramount considerations. In these countries, nalidixic acid (first-generation quinolone) remains a valuable alternative (Germani et al., 1998b).

Travelers are infected with multiresistant *Shigella* with increasing frequency (Heikkila et al., 1990; Tauxe et al., 1990). Recent data from the National *Shigella* Reference Center in Finland (Heikkila et al., 1990) showed that 98% of strains isolated from travelers were resistant to trimethoprim, whereas only 3% of the strains were resistant to this antibiotic before 1975. In the United States, among domestically acquired isolates, only 5% are resistant to trimethoprim-sulfamethoxazole and 10% to ampicillin, whereas among isolates collected from travelers, nearly 20% are resistant to trimethoprim-sulfamethoxazole and 60% to ampicillin (Tauxe et al., 1990). During operation Desert Shield/Storm in the Arabian Peninsula (Hyams et al., 1991), 85% of the *Shigella* strains tested were resistant to trimethoprim-sulfamethoxazole. Among strains isolated from soldiers in the operation in Somalia in 1995 (Sharp et al., 1995), a high level of resistance to doxycycline, ampicillin and trimethoprim-sulfamethoxazole was reported.

The suggestion to reserve antibiotic treatment to the most severe cases of shigellosis is also a matter of debate (Wennerås et al., 2000; Weissman et al., 1973). A reason to limit the use of antibiotics in this case is that they have been implicated in the development of the hemolytic uremic syndrome (Butler et al., 1987). This seems to be unlikely, as the most severely affected patients are the most likely to both receive antibiotics and develop severe systemic complications.

## Specific Immune Response Against *Shigella* Infection

Most of our knowledge of the specific (adaptive) immune response against *Shigella* has been collected from observations in humans. Evidence indicates that *Shigella* infection confers protective immunity, although its mechanisms are not fully understood. Among the evidence is the fact that shigellosis peaks during the first five years

of life and subsequently declines, suggesting that immunity occurs following repeated exposures to *Shigella* during childhood (Taylor et al., 1986). Also, the incidence of the disease decreases with the duration of stay in an endemic setting (Cohen et al., 1992). Following *Shigella* infection, protection against the homologous serotype is established (DuPont et al., 1972). This suggested that LPS was the major target of protection and that the humoral, likely local, immunity was the major effector. Strong evidence for serotype-specific natural immunity was provided by a study in Chilean children in whom the primary *Shigella* infection conferred 76% protective efficacy against reinfection with a homologous serotype (Ferrecio et al., 1991). Serum and stool antibody levels of anti-*Shigella* LPS and Ipa proteins have been detected following natural infection and infection in volunteers (Cam et al., 1993; Islam et al., 1995; Oberhelam et al., 1991; Cohen, et al., 1989). Specific anti-LPS IgA antibodies in stool samples peak within 5–10 days of infection and may participate in resolution of infection, taking over the attempt of the innate response to eradicate shigellae (Cam et al., 1993). In parallel, increasing levels of serum anti-LPS IgG and IgA also are observed. Stool and serum IgA levels then fall rapidly to background level within 2–3 months. People living in areas where shigellosis is endemic have higher background serum IgG levels and mount greater serum IgG response than infected people living in nonendemic areas. On the other hand, mucosal IgA levels appearing in response to infection are similar in both groups. These results suggest that a systemic IgG memory response exists, whereas the mucosal IgA memory response is of relatively short duration.

The murine pulmonary model of shigellosis also has been used to study the specific immune response, although the local IgA and IgG responses against LPS and Ipa proteins following a sublethal infection are slow to develop and need a second challenge (Van De Verg et al., 1995). Short-lived serotype-specific protection nevertheless was demonstrated in this model; although IgM seemed to be predominantly involved, T-cell mediated protection did not seem to play a role. This model also has been used to demonstrate that anti-LPS IgA can protect against challenge with a lethal dose of *Shigella* (Phalipon et al., 1995).

In summary, protective immunity seems to be essentially dependent on a local humoral response against LPS, although this local immune memory appears short lived compared to the serum IgG response whose actual protective capacity is neither sufficiently evaluated nor clearly explained. In addition, although it is difficult to believe that an infection that has a clear

intracellular stage does not involve a strong protective T-cell component, the actual function of cellular immunity in shigellosis remains unknown.

## Prevention of *Shigella* Infections

Because the only significant source of *Shigella* infection is infected humans and the transmission most often is oro-fecal, sanitary measures are essential for disease prevention. These measures should include hand washing with soap, chlorination of water, proper disposal of feces, and protection of food from contamination, particularly by flies. Patients, particularly those preparing foods, should be isolated ideally until their stool cultures have become negative. Proper antibiotherapy, which shortens the duration of *Shigella* excretion, also may help decrease the spread. However, control may not be easy in the most impoverished regions in which the disease is endemic, or in emergency situations (i.e., constitution of refugee camps) where implementation of proper personal and general hygiene standards is difficult. This may be complicated by the high incidence of unapparent infections in such situations and the low inoculum of shigellae, which is required to elicit the disease. These considerations, added to the increased resistance to antibiotics, have led to consideration of vaccination against shigellosis as an efficient and cost-effective approach for prevention.

## Toward Vaccine Prevention

Considering that the protective immunity against *Shigella* infection is essentially serotype-specific, the protective capacity of any *Shigella* vaccine candidate will depend greatly on the representation of serotypes incorporated in the vaccine and the epidemiological importance of these respective serotypes in the area. This emphasizes the need for evaluation of the disease burden and of the representation of serotypes in any area where such vaccine candidates are to be studied (Clemens et al., 1999).

Initial attempts at immunizing against shigellosis were based on whole-cell killed preparations administered parenterally. The complete failure that followed this approach led to the concept that parenteral immunization could not successfully protect against shigellosis. For a disease that remained local, mucosal immunization with live attenuated oral vaccine candidates was a better option. Initial attempts in the mid-sixties using multiple oral doses of streptomycin-dependent (SmD) strains provided very encouraging results and also indicated that the

protection that could be achieved was serotype-dependent (Mel et al., 1965). In addition to the preparation of subcellular vaccines such as proteosomes and ribosomes, which still need to demonstrate protective capacity in humans, two major approaches have been investigated recently with encouraging results (Sansonetti, 1998).

A subunit parenteral vaccine composed of purified/detoxified *S. sonnei* LPS conjugated with a toxoid has shown about 70% homologous protection in the course of a *S. sonnei* outbreak in the Israeli Army (Cohen et al., 1996). Unlike killed whole-cell vaccines, these polysaccharidic conjugates elicit high anti-polysaccharide IgG titers that may protect via diffusion through the colonic epithelial barrier.

Alternatively, different groups have engineered several live-attenuated vaccine candidates. Attenuation strategies are based on the deletion of gene(s) involved in encoding key enzymes of metabolic pathways that are essential for bacterial survival in tissues (i.e., mutations in *aro* genes or *guaB*, A genes) and/or pathogenicity genes that are essential for the bacterium to proceed to a key step of its pathogenic program (i.e., the *icsA* gene). So far, a *S. flexneri* 2a mutant (SC602) that has undergone a deletion in both *icsA* and *iuc* (the operon encoding the Fe<sup>3+</sup> chelator aerobactin) has been shown to be protective after a single oral dose against a homologous virulence strain in a challenge study in volunteers (Coster et al., 1999). Further studies are going on in endemic areas. Similar strains have been constructed in *S. dysenteriae* 1 and *S. sonnei*.

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## The Genus *Salmonella*

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### Introduction and History

The *Salmonella* live in the intestines of animals and have evolved with their hosts (see Phylogeny). Thus, *Salmonella* infection is an ancient disease, and typhoid fever has been well described throughout written history (Cunha, 2004). However, confusion between the diseases typhus and typhoid existed until the mid-1800s, even though previous investigators had differentiated the two (Miller and Pegues, 2000; Cunha, 2004). For example, Oxford physician Thomas Willis (1621–1675), in 1659, noted the ileal ulcers associated with typhoid. With the increasing use of autopsies, in the 1820s, Pierre Bretonneau (1778–1862) and colleagues described that the Peyer's patches were inflamed in typhoid. He also noted that the disease was contagious and that once a person survived typhoid, they did not get the disease again. French physician Pierre Charles Alexandre Louis (1787–1872) first used the term “typhoid fever” in 1829. William Jenner (1815–1898) further clarified the differences between typhus and typhoid based on symptomology and, importantly, epidemiology in 1850; the fact that he personally suffered from both diseases strengthened his differential diagnosis. William Budd (1811–1880) concluded in 1873 that typhoid fever was spread by the fecal-oral route. Karl Eberth (1835–1926) observed in 1880 rod-shaped organisms in the spleens and lymph nodes of typhoid patients and is credited with discovering the serovar Typhi organism. Georg Gaffky (1850–1918) first successfully cultured serovar Typhi from patients in Germany in 1884. In 1885, Theobald Smith (1859–1934), working under American veterinarian Daniel E. Salmon (1850–1914), isolated what became known as *Salmonella choleraesuis* from the intestine of a pig. French bacteriologist Joseph Léon Marcel Lignières (1868–1933) suggested in 1900 that the group of bacteria represented by the swine-cholera organism should be termed “*Salmonella*” in honor of Salmon. Georges Widal (1862–1929) in 1896 coined the term “agglutinin” to describe the clumping of heat-killed serovar Typhi cells by convalescent serum (the Widal reaction).

Almroth Edward Wright (1861–1947) and, independently, Richard F.J. Pfeiffer (1858–1945) and Wilhelm Kolle (1868–1935), used heat-killed organisms to vaccinate against typhoid in 1896; essentially the same vaccine is still in use today (see Serovars Typhi and Paratyphi). Elie Metchnikov (1845–1916) fulfilled Koch's postulates to prove that serovar Typhi caused typhoid in 1911. Fritz Kauffmann (1899–1978), extending the work of P.B. White (White, 1926), established serological analysis of *Salmonella* starting in the 1940s. In 1948, Theodore E. Woodward (1914–) and colleagues successfully treated patients in Malaysia with chloromycetin (chloramphenicol).

Serovar Typhimurium strains became a favorite tool of the burgeoning number of bacterial geneticists. In 1948, K. Lilleengen characterized a series of 25 clinical isolates representing different phage sensitivities (see Phage Typing). He designated these strains “Lilleengen Type” or LT1 through LT25 (Lilleengen, 1948). Serovar Typhimurium strain LT2 is now the type strain (see Taxonomy) and the complete genome has been sequenced (McClelland et al., 2001). Norton D. Zinder (1928–) and Joshua Lederberg (1925–) used the LT strains to study genetic exchange, leading to the discovery in 1952 of bacteriophage-mediated transduction (Zinder and Lederberg, 1952). The phage in question was P22 (isolated from LT22), the workhorse of *Salmonella* genetics. Milislav Demerec (1895–1966) obtained the LT strains from Zinder and began a series of genetic studies. The resulting LT2 (and LT7) derivatives are still being characterized (Rabsch et al., 2004). Indeed, LT2 rivals *Escherichia coli* K12 as an object of genetic analysis (see <http://www.ecosal.org>). This chapter covers aspects of the *Salmonella* genus, mainly recent studies and recent reviews of the major topics. The reader is directed to these reviews and the references therein.

### Phylogeny

*Salmonella* are members of the Enterobacteriaceae, in the Gamma-proteobacteria subdivi-

sion. Two species of *Salmonella* are recognized: *Salmonella bongori* and *Salmonella enterica*. *Salmonella enterica* can be further divided into seven subspecies on the basis of biochemical characteristics (biotype), differences observed in multilocus enzyme electrophoresis (MLEE), phylogenetic analysis using 16S rRNA or other sequences, or analyses using other molecular techniques such as amplified-fragment length polymorphism (AFLP; Selander et al., 1996). Members of *S. enterica* subsp. I account for 99% of all human infections. Molecular analyses of *Salmonella* have been facilitated by the existence of the “*Salmonella* reference collections” (available from the *Salmonella* Genetic Stock Center). *Salmonella* reference collection A (SARA) contains 72 strains from a variety of environments and hosts (Beltran et al., 1991). SARB consists of 72 strains of *S. enterica* subsp. I (Boyd et al., 1993), while SARC contains two strains of *S. bongori* and two strains from each of the seven subspecies of *S. enterica* (Boyd et al., 1996). Strains in all three collections have been well characterized by Selander and colleagues using MLEE.

The availability of complete genomic sequences (McClelland et al., 2001; Parkhill et al., 2001) has significantly increased our understanding of the evolution of *Salmonella* and their relation to other members of the Enterobacteriaceae. McClelland and colleagues compared the genetic content of strains in the SARC collection and other representatives of the Enterobacteriaceae by direct probing of a *Salmonella enterica* serovar Typhimurium strain LT2 open reading frame (ORF) microarray with genomic DNA from the strain of interest, or comparison of completed genomic sequences (Porwollik et al., 2002). The overall results of this analysis in the form of a phylogenetic tree are presented in Fig. 1. Consistent with previous studies (Baumler et al., 1998), these data suggest that the evolution of *Salmonella* has been largely dictated by the acquisition of horizontally transferred segments of DNA, or islands, encoding virulence factors that have allowed the new subspecies to colonize a different niche.

A general model for the evolutionary history of the genus, delineated by a variety of investigators (Selander et al., 1996; Baumler et al., 1998; Edwards et al., 2002; Porwollik et al., 2002; Chan et al., 2003) can be summarized as follows (Fig. 1). *Salmonella* and *E. coli* diverged 120–160 million years ago, about the time that mammals first appeared. Approximately 25–40 million years ago, the common *Salmonella* ancestor acquired the *Salmonella* pathogenicity island 1 (SPI1) which encodes a type III secretion system involved in invasion of the intestinal epithelium (see SPI1 TTSS). Thus, *Salmonella* became an

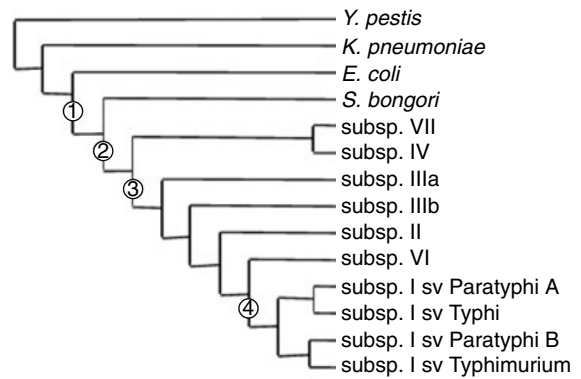


Fig. 1. Model for the evolution of *Salmonella*. 1) The common *Salmonella* ancestor acquired the SPI1 type III secretion system (TTSS) allowing intestinal invasion. 2) The two species of *Salmonella* diverged when the *Salmonella enterica* ancestor acquired the SPI2 TTSS allowing invasion of deeper tissue. 3) *Salmonella enterica* subsp. I, II, IIIa, IIIb, IV, VI and VII acquired the ability to phase shift multiple flagellin subunits, thereby avoiding the immune response. 4) The subspecies continued to differentiate in their respective niches with subsp. I strains acquiring the ability to colonize warm-blooded host. Adapted from Baumler et al. (1998) and Porwollik et al. (2002).

intracellular pathogen associated with cold-blooded vertebrates. The next major event was the acquisition of the *Salmonella* pathogenicity island 2 (SPI2) type III secretion system that allows the organisms to survive in macrophages and, thus, colonize deeper tissue (see SPI2 TTSS). This event resulted in the split between the two currently recognized species, the presence of SPI2 being a defining characteristic of *S. enterica*. The seven subspecies of *S. enterica* (I, II, IIIa, IIIb, IV, VI and VII) continued to evolve to fit their respective niches. The ancestor of subsp. I, II, IIIb and VI acquired the ability to “phase shift” between multiple structural subunits of flagella, the primary organ of motility (see Flagella). This apparently provided *S. enterica* with a further ability to evade the immune systems of their hosts. The subsp. I ancestor then acquired the ability to colonize warm-blooded vertebrates, a major advance with striking implications for human health. The subsp. I strains have subsequently evolved to colonize a variety of hosts. Some serovars, such as Typhi, are host-specific, infecting only humans, whereas serovar Typhimurium is a generalist, capable of colonizing and causing disease in a range of mammalian species.

Chan et al. (2003) also analyzed subsp. I, IIIa and *S. bongori* strains using a serovar Typhimurium microarray. Although their results are in general agreement with those of Porwollik et al. (2002), important differences in interpretation exist between the two research groups. Indeed,

Chen et al. suggest that *S. enterica* subsp. IIIa (serovar *Arizonae*) is the most divergent of the *Salmonella*, with *S. bongori* being intermediate between subsp. IIIa and other *S. enterica* strains. Although the subsp. IIIa strains examined hybridized to some SPI2 gene probes, the pattern was heterogeneous. As described, *S. bongori* is apparently missing SPI2, which distinguishes it from *S. enterica* strains. Chan et al. raise the possibility that both *S. bongori* and *S. enterica* subsp. IIIa strains are derived from SPI2-containing ancestors but have lost some or all of these genes during their adaptation to their respective hosts.

Although the major island encoded virulence factors appear to delineate the evolution of the subspecies, they are certainly not the only genes specific to the various evolutionary branches. (See McClelland et al. [2001], Parkhill et al. [2001], Porwollik et al. [2002], Edwards et al. [2002], and Deng et al. [2003] for a more complete discussion of genes specific to *Salmonella* serovars.) Exchange of genetic material is taking place constantly. Whether or how much each of these subspecies-specific genes contributes to virulence or host range is an area of intense study. Alternatively, these genes could have simply been present in a particular strain at the moment it gained a major virulence factor that allowed it to take over the population. Throughout the evolution of *Salmonella*, certain strains acquired traits that made them significantly more fit or able to colonize a new niche, thus explaining the clonality of the various subspecies.

Some genes and traits are more highly variable than the bulk of the genome. This is particularly true for gene products that are subject to immunological selection, including fimbriae, O antigen, and flagella. Becoming increasingly clear is that many virulence factors are carried on bacteriophage that are lysogenized into the chromosome of particular strains (Thomson et al., 2004). For example, the Gifsy-2 phage encodes two major virulence factors, as well as other proteins that clearly interact with the host (Figueroa-Bossi and Bossi, 1999; Ho et al., 2002). Gifsy-2 lysogens are 100-fold more virulent in a mouse model of infection than an isogenic non-

lysogen (Ho et al., 2002). However, the presence of Gifsy-2 in different serovars of *Salmonella* is apparently unpredictable, being present in some isolates of certain serovars while absent in others (Fang et al., 1999; Ho and Schlauch, 2001). This observation is likely due to the fact that Gifsy-2 is a viable phage. Thus, “horizontal gene transfer” is constantly taking place. In other words, the presence or absence of the phage in a given isolate is not a reflection of the evolutionary history of the entire serovar.

## Taxonomy

*Salmonella* nomenclature is undergoing constant change and can be confusing (Brenner et al., 2000; Euzéby, 2000). Until the mid-1900s *Salmonella* “species” were descriptive of the associated disease, e.g., *Salmonella typhi*, *Salmonella choleraesuis*, *Salmonella typhimurium*. Kauffmann (Kauffmann, 1941; Kauffmann, 1966; Kauffmann, 1971), extending the work of White (1926), established serological analysis of *Salmonella* (see Identification). Kauffmann argued that antigenic formula was “as good or even much better” than a species name (Kauffmann, 1971), thus serotype became equated with species. Newer isolates are named after the site of isolation, e.g., *Salmonella london*, *Salmonella paris* and *Salmonella casablanca*. Kauffmann, however, established “subgenera” based on biochemical characteristics. Contrary to his intention (Kauffmann, 1971), these subgenera also became equated with species, e.g., *Salmonella salamae* for subgenus II (Le Minor and Popoff, 1987). In 1966, the Ninth International Congress for Microbiology condensed the older names, for example, *Salmonella typhimurium*. There are currently over 2500 documented *Salmonella* serotypes (Popoff et al., 2001b).

Molecular studies suggested that all *Salmonella* are closely related (see Phylogeny), and Le Minor, Popoff, and colleagues (Le Minor, 1982; Le Minor, 1986) proposed the single species name *Salmonella choleraesuis* with seven recognized subspecies (Table 1). Subspecies I strains have recognized serovars, e.g., *Salmonella choleraesuis* subsp. *choleraesuis* serovar Typhi-

Table 1. *Salmonella* nomenclature.

Subspecies	Approved nomenclature	Widely accepted nomenclature
I	<i>Salmonella choleraesuis</i> subsp. <i>choleraesuis</i>	<i>Salmonella enterica</i> subsp. <i>enterica</i>
II	<i>Salmonella choleraesuis</i> subsp. <i>salamae</i>	<i>Salmonella enterica</i> subsp. <i>salamae</i>
IIIa	<i>Salmonella choleraesuis</i> subsp. <i>arizonae</i>	<i>Salmonella enterica</i> subsp. <i>arizonae</i>
IIIb	<i>Salmonella choleraesuis</i> subsp. <i>diarizonae</i>	<i>Salmonella enterica</i> subsp. <i>diarizonae</i>
IV	<i>Salmonella choleraesuis</i> subsp. <i>houtenae</i>	<i>Salmonella enterica</i> subsp. <i>houtenae</i>
VI	<i>Salmonella choleraesuis</i> subsp. <i>indica</i>	<i>Salmonella enterica</i> subsp. <i>indica</i>
V	<i>Salmonella choleraesuis</i> subsp. <i>bongori</i>	<i>Salmonella bongori</i>

murium or *Salmonella choleraesuis* subsp. *choleraesuis* serovar Typhi (note the use of italics and capitalization). These subspecies names currently have standing on the Approved Lists (Anonymous, 2003). The names *Salmonella arizonae*, *Salmonella bongori*, *Salmonella enteritidis*, *Salmonella typhi* and *Salmonella typhimurium* are also approved (see Genus *Salmonella* – Bacterial Nomenclature Up-to-Date).

Noting the potential confusion in using *Salmonella choleraesuis* to indicate organisms that cause very different diseases, e.g., serovar *Choleraesuis* versus serovar Typhi, Le Minor and Popoff (1987) proposed that the name *Salmonella enterica* be the sole species designation (Table 1) and that a particular stock of *Salmonella enterica* subsp. *enterica* (I) serovar Typhimurium strain LT2 be the type strain. The LT2 strain is Lilleengen strain type 2 (Lilleengen, 1948) and is extremely well characterized at the genetic, biochemical and molecular level. The proposal had received unanimous support from the Subcommittee on Enterobacteriaceae of the International Committee on Systematic Bacteriology at the Fourteenth International Congress of Microbiology (Penner, 1988). Subsequently, Reeves et al. (1989) proposed that *Salmonella bongori* be elevated to species status on the basis of molecular characterization. Although these proposals have never been officially sanctioned, this nomenclature is used widely and has been adopted by the Centers for Disease Control and Prevention (CDC), the American Society for Microbiology, and the WHO Collaborating Centre for Reference and Research on *Salmonella*, which is responsible for updating the Kauffmann-White Scheme (Popoff and Le Minor, 1997; Popoff et al., 2001b). Serovars of *Salmonella enterica* subsp. *enterica* are written in Roman letters (not italicized) and the first letter is capitalized. Serovars of other subspecies are designated by their antigenic formulae (see Identification). Since only *Salmonella enterica* subsp.

*enterica* strains are named, various shortened versions have become acceptable, such as *Salmonella enterica* serovar Typhimurium, *Salmonella* Typhimurium, or serovar Typhimurium. The *Salmonella* are also classified on the basis of serological differentiation of both O-antigen and flagellar antigens. This system and its nomenclature are described in Identification (see also Brenner et al., 2000).

## Habitat

The *Salmonella* live primarily in the intestinal tracts of animals. *Salmonella enterica* subsp. *enterica* inhabit warm-blooded animals, whereas all other *S. enterica* subspecies and *S. bongori* are commensals of cold-blood animals and only rarely infect humans. The *S. enterica* subsp. *enterica* serovars can be further divided into those that are “host adapted” (primarily infecting one host but capable of causing disease in others), “host restricted” (known to infect only a single host), and “generalists” (capable of infecting a variety of animals, although the course of disease may differ in different hosts; Uzzau et al., 2000; Edwards et al., 2002; Table 2). Our understanding of the host range of *Salmonella* and the interaction of specific serovars with their host(s) is clearly affected by our preoccupation with human health; the nonhuman hosts listed in Table 2 are mostly food production animals or, in the case of rodents, responsible for contamination of food production facilities or involved in research. The real host range and distribution of *Salmonella* could be much larger.

*Salmonella* infection of many of these hosts often leads to a self-limiting gastroenteritis (see Disease and Gastroenteritis). However, to be maintained in its habitat, *Salmonella* must be able to persist or live commensally with at least some percentage of hosts and be efficiently transmitted from host to host. The molecular mecha-

Table 2. Host range of *Salmonella enterica* subsp. *enterica* serovars.

Classification	Serovar	Natural host	Rare hosts
Host restricted	Typhi	Humans	None
	Paratyphi A and C	Humans	None
	Sendai	Humans	None
	Abortusovis	Ovines	None
	Gallinarum	Poultry	None
	Typhisuis	Swine	None
	Abortusequi	Equines	None
Host adapted	Choleraesuis	Swine	Humans
	Dublin	Bovines	Human and ovines
Generalist	Typhimurium	Humans, poultry, swine, bovines, and rodents	None
	Enteritidis	Humans, poultry, and rodents	Swine and bovines

Adapted from Uzzau et al. (2000) and Edwards et al. (2002).



nisms of persistence are not well understood, and multiple systems are likely to contribute in the various serovars (Kingsley et al., 2003). Clearly, persistence and dissemination are genetically defined, as evidenced by the recent worldwide emergence of particular strains, such as serovar Typhimurium DT104 (Davis et al., 2002) and serovar Enteritidis PT4 (Guard-Petter, 2001).

Dissemination also requires that *Salmonella* survive outside of animals, and organisms can clearly be isolated from environmental sources. Contaminated water, for example, is a significant source of serovar Typhi in endemic areas (see Epidemiology). Infection of animals and food-stuffs is via contaminated feces. Clearly, *Salmonella* can replicate in contaminated food that is rich in nutrients and stored improperly. However, whether the bacteria are replicating or simply surviving in other environments is not clear. For example, because they filter large volumes of water to feed, mollusks can concentrate *Salmonella* that have contaminated coastal waters from sewage effluent and these contaminated shellfish can be a significant source for human infection (Noble, 1990). When oysters that were experimentally infected with serovar Typhi or serovar Typhimurium were placed in clean water, the number of bacteria associated with the shellfish was rapidly reduced, suggesting that the bacteria do not actually colonize and grow in the live shellfish (Hartland and Timoney, 1979; Nishio et al., 1981). Likewise, Gray and Fedorka-Cray (2001) have shown that serovar Choleraesuis survives for months and remains infective in swine feces. However, the number of recoverable cells consistently declined with time in these environments, suggesting that, although the organisms can survive, they do not replicate significantly.

Two cases where *Salmonella* has adapted to particular niches to maintain itself in the host population are particularly noteworthy. Serovar Typhi is a host-restricted human pathogen. Infection, particularly in children, results in an enteric fever (see Disease). Transmission is by the fecal-oral route. The bacteria invade the intestinal epithelium and invade macrophages. The bacteria replicate in macrophages and are disseminated throughout the body. Ninety percent of untreated Typhi patients who survive will completely clear the infection within several weeks. However, 10% will shed bacteria for up to 3 months, and 1–4% become long-term carriers, producing infective bacteria in their stools for over a year (Miller and Pegues, 2000; Parry et al., 2002). Bacteria replicating in the liver are shed through the gallbladder into the intestine and, thereby, the feces. Both acute (cholecystitis) and chronic infection of the gallbladder can develop. These chronic infections are more common in women and the elderly and are associated

with gallstones. It has been suggested that serovar Typhi can form biofilms on the gallstones, providing a more permanent habitat (Prouty et al., 2002a; see Genetics). Note that these carriers are asymptomatic, and many have no apparent history of typhoid fever but provide a constant source of infectious bacteria. The most famous carrier was “Typhoid Mary” (Leavitt, 1996).

Serovar Enteritidis is a generalist, capable of causing disease in a variety of hosts. Indeed, this serovar competes with serovar Typhimurium as the leading cause of human infections (see Epidemiology). In chickens, however, serovar Enteritidis causes an asymptomatic infection of the hen reproductive tract. This leads to vertical transmission, where the bacteria contaminate the egg prior to the deposition of the shell (Guard-Petter, 2001). Thus, these otherwise healthy hens provide a long-term source of *Salmonella*.

## Isolation

The isolation of *Salmonella* spp. from food products utilizes a selective enrichment method (Harrigan, 1998). Samples, after resuscitation in, for example, peptone water, are used to inoculate selenite cystine broth or Rappaport-Vassiliadis medium, both which enrich for *Salmonella* and *Shigella*. Selenite cystine broth is commonly used for enrichment of *Salmonella* because selenite inhibits growth of most coliforms but has only mild effects on *Salmonella*. The resulting cultures are then plated on selective and differential media such as brilliant green phenol red agar or bismuth sulfite agar. Once putative single colonies of *Salmonella* have been isolated, biochemical and serological methods can be used to definitively determine whether the samples indeed contain *Salmonella* (see Identification).

The isolation of *Salmonella* from clinical samples, such as stool samples, involves the use of a nonselective medium such as blood agar, or selective and differential media such as MacConkey or a highly selective medium such as bismuth sulfite agar (Farmer, 1999). Bismuth sulfite agar is particularly useful in identifying *Salmonella* because bismuth, a heavy metal, inhibits the growth of many other microorganisms (Farmer, 1999). On bismuth sulfite agar, most *Salmonella* (Table 3) appear black because of the production of hydrogen sulfide, which reacts with ferrous sulfate to form the black precipitate. The sensitivity of culturing stool for *Salmonella* is estimated to be 70% (Voetsch et al., 2004).

Recently a great deal of interest has focused on the presence of nonculturable but viable *Salmonella* species in clinical, environmental, and food samples. Recent studies suggest that the addition of certain supplements increases

Table 3. Biochemical characteristics of selected Enterobacteriaceae.

Test	<i>Salmonella enterica</i>																			
	Subspecies Serovar	Typhimurium	Typhi	Paratyphi A	Choleraesuis	Pullorum	Gallinarum	II	III a or b	IV	VI	V Bongori	Citrobacter freundii	<i>E. coli</i>	<i>Edwardsiella</i> <i>urtada</i>	<i>Klebsiella</i> <i>pneumoniae</i>	<i>Proteus</i> <i>mirabilis</i>	<i>Shigella</i> <i>sonnei</i>	<i>Yersinia</i> <i>enterocolitica</i>	
Growth on MacConkey agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	-	-	-
Growth in the presence of KCN	-	-	-	-	-	-	-	-	-	-	-	+	+/-	-	-	+	+	-	-	-
Metabolism of glucose																				
Fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas production	+	-	+	+	+	+	-	+	+	+	+/-	+	+	+	+	+	+	-	-	-
Acetoin production (Voges-Proskauer)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	I	-	-	-
Production of																				
Arginine dihydrolase	+/-	-	-	-/+	I	-	-	-	+/-	+/-	+	+	I	-/+	-	-	-	-	-	-
Cytochrome oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	-
H <sub>2</sub> S	+	+	-	-	I	+	+	+	+	+	+	+	+/-	-	+	-	+	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	I
Lysine decarboxylase	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-
Nitrate reductase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	-	-	+	+	+	-	+	+	+	+	+	-	I	+	-	+	+	+	+
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	+	+	-	-	+



the recovery of stressed but otherwise nonculturable cells. These supplements include 1) the siderophore ferrioxamine E, 2) the commercially available antioxidant “Oxyrase,” and 3) heat stable enterobacterial “autoinducer” (Reissbrodt et al., 2002).

## Identification

Proper identification of *Salmonella* requires isolation, biochemical characterization, and serotyping. The *Salmonella* are members of the Enterobacteriaceae: Gram-negative, oxidase-negative, rod-shaped bacteria (1 · 2 μm). Most *Salmonella* strains are motile and ferment glucose with the production of acid and gas. Table 3 lists some useful biochemical tests to distinguish *Salmonella* from other enterics and to differentiate *Salmonella* subspecies. Many of these tests are most easily carried out using commercially available systems, such as API-20E (bioMérieux, Inc.) or BBL Enterotube II (BD; see O’Hara et al. [2003] for more information).

*Salmonella* are serotyped on the basis of the antigenic structure of the O antigen (heat stable somatic antigen; see Structure), the H antigen (heat labile; flagellin; see Structure)), and the Vi antigen (capsule; see Structure), if present. Serotyping is only valid on strains that are biochemically confirmed as *Salmonella* because the antisera are crossreactive with other bacteria. The serotypes are designated according to the Kauffmann-White scheme (Popoff, 2001a). Note that the CDC used a “Modified Kauffmann-White Scheme” before January 2003 but now uses the standard scheme.

## O Antigen

Lipopolysaccharide (LPS) forms the outermost layer of Gram-negative bacteria and serves to protect the cell from the environment. LPS is composed of three portions: lipid A, core oligosaccharide, and a polymer of O-antigen subunits (Raetz and Whitfield, 2002). In pathogenic bacteria, LPS plays an important role in the interaction between the bacterium and its host, having dramatic effects on the immune system. Whereas the lipid A moiety is the predominant cause of the endotoxic effects of LPS, the O antigen is the most immunodominant portion of the molecule.

Because of selection due to the immune system of *Salmonella* hosts (Kingsley and Baumler, 2000), bacteriophages (which often use O-antigen as a receptor), and protozoal predation (Wildschutte et al., 2004), the structure of the O-antigen is very variable among strains of *Salmo-*

*nella*. Structurally, the O antigens can differ in monosaccharide components, linkages between monosaccharides, and other minor modifications, such as acetylation. Even subtle chemical changes in O-antigen structure have profound effects on antibody recognition (Kim and Slauch, 1999). Thus, the Kauffmann-White scheme recognizes O-antigen factors 1–67 (not inclusive) and divides the *Salmonella* into approximately 50 different serogroups (Table 4). As examples, the basic structure of the O-antigen subunits for groups B and D1 and common modifications are shown in Fig. 2.

The enzymes required for synthesis of the O-antigen specific monosaccharides and assembly of the subunit are located in the O-antigen gene cluster at approximately centisome 45 on the serovar Typhimurium strain LT2 chromosome (McClelland et al., 2001). Not surprisingly, comparison of the gene cluster among different serovars of *Salmonella* shows considerable variation (Wang et al., 2002). Genes encoding the synthesis of more common monosaccharides (e.g., galactose) are located elsewhere in the chromosome, while many of the enzymes that modify the O antigen are carried on viable or cryptic prophages (Slauch et al., 1996). The presence or absence of these modifications is often variable within a serogroup.

Table 4 lists the O factors that comprise the different O-antigen groups. The majority of human isolates are in serogroups A–E4. However, serogroup and subspecies are not strictly correlated (see Taxonomy), i.e., subspecies I strains are found in all serogroups. Strains are initially tested against antisera for Groups A–E4. Positive results are confirmed using the appropriate antisera against the single factors that will distinguish between groups. If agglutinations with Groups A–E4 antisera are negative, other Group antisera are tested, usually in pools, e.g., antisera against Groups 51–55. (In-depth technical information about O-antigen serogrouping is found in Brenner and McWhorter-Murlin [1998] and Difco [1998].)

If no agglutination occurs with all group antisera, then possibly the strain (e.g., serovar Typhi) is producing Vi capsule that is blocking antibody access to the O antigen (see Vi Antigen). Previous biochemical analysis should suggest serovar Typhi strains. Alternatively, the strain could be “rough” and is not making O antigen. These strains will either not agglutinate or give weak cross-agglutination in a variety of antisera. The negative strain could be “mucoid” and produce a capsule other than Vi. Both rough and mucoid strains can be streaked out and typical colonies can be identified and tested. Finally, the strain might not be *Salmonella*. Biochemical tests should be confirmed.

Table 4. O-antigen factors present in O-antigen groups.

O antigen group	O antigen factors present <sup>a</sup>
A	<u>1</u> , 2, 12
B	4, 12; <u>1</u> , 4, 12; <u>1</u> , 4, 5, 12; or <u>1</u> , 4, 12, <u>27</u>
C1	6, 7, [Vi] or 6, 7, <u>14</u>
C2	6, 8
C3	8; or 8, <u>20</u>
D1	<u>1</u> , 9, 12, [Vi]
D2	9, 46
D3	1, 9, 12, 46, 27
E1	3, 10
E2	3, <u>15</u>
E3	3, <u>15</u> , <u>34</u>
E4	1, 3, 19
F	11
G	13, 22 or 13, 23
H	6, 14; 6, 14, 24; or 1, 6, 14, 25
I	16
J	17
K	18
L	21
M	28
N	30
O	35
P	38
Q	30
R	40
S	41
T	42
U	43
V	44
W	45
X	47
Y	48
Z	50
51	51
52	52
53	53
54	54
55	55
56	56
57	57
58	58
59	59
60	60
61	61
62	62
63	63
65	65
66	66
67	67

<sup>a</sup>Underlined antigens are conferred by lysogenic phage. Those in brackets are also variable but not known to be conferred by viable phage.

Adapted from Brenner and McWhorter-Murlin (1998) and Difco (1998).

## H Antigens

Most *Salmonella* strains are motile, producing peritrichous flagella. The flagella are exposed on the surface of the bacteria and are highly immunogenic. Therefore, as in the case of O antigen,

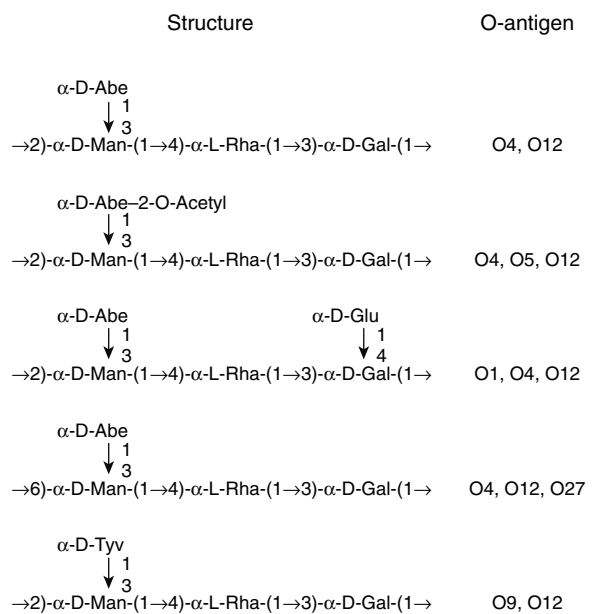


Fig. 2. Structures of representative O antigens. The basic structure (e.g., O4 and O12) can be modified. The O5 antigen is the result of acetylation of the 2-hydroxyl group on abequeose, encoded on a cryptic bacteriophage (Slauch et al., 1996). The O1 antigen is a phage-mediated addition of glucose on the galactose. The O27 epitope results from altered linkage between the O-antigen subunits (Wang et al., 2002). The difference between O4 and O9 is the use of abequeose versus tyvelose attached to the mannose residue. Adapted from D. Liu et al. (1995) and Wang et al. (2002).

there has been selection for considerable variation in the surface-exposed domains of the flagellar subunit. Indeed, subsp. I, II, IIIa and VI are biphasic, capable of producing two (and sometimes three) functionally equivalent but immunologically distinct flagellar subunits (see both Phylogeny and Flagella). Serological distinction among the H antigens constitutes the second part of the Kauffmann-White scheme.

Phase 1 H antigens are designated by lowercase letters, "a" through "z" and then "z1," "z2," "z3," etc. Phase 2 antigens are designated by numerals, "1," "2," "3," etc. However, some strains produce Phase 1 antigens in Phase 2. H antigens are identified only after the O-antigen group has been determined; particular H antigens are associated with certain serogroups. H antigens are usually determined using a tube agglutination assay. Initially, agglutination with mixed unabsorbed H antisera is performed. These pools are designed to be used with certain O-antigen groups as they represent the most common H antigens associated with these groups. Further identification requires the use of absorbed single H antisera. (In depth technical information about H-antigen serotyping is found in Brenner and McWhorter-Murlin [1998] and Difco [1998].) The Spicer-Edwards H antisera

are combined sera designed to easily distinguish the most common Phase 1 antigens (Difco, 1998).

Within the culture of most strains, both Phase 1 and 2 H antigens will be expressed. However, fresh single colony isolates may only express one phase. To determine the other phase, one can perform a “phase reversal.” This is accomplished by inoculating the strain of interest into motility agar that contains H antiserum that is known to agglutinate. The antibodies in the serum will immobilize cells expressing the original H-antigen (at any given time, one antigen being expressed) but not cells that have undergone a phase transition and so are expressing a new antigen. The progeny of the latter will swim away from the site of inoculation. These cells can be isolated and the new H antigen determined. If the strain does not give rise to motile cells in the presence of the H antiserum, it could be monophasic.

### Vi Antigen

The Vi antigen is a capsular polysaccharide produced by serovar Typhi and Paratyphi C strains, some *Citrobacter* strains, and occasionally by strains of serovar Dublin. The capsule is a linear homopolymer of  $\alpha$ -1,4 2-deoxy-2-*N*-acetylgalactosamine uronic acid which can be O-acetylated at the C-3 position (Daniels et al., 1989). The enzymes required for synthesis and export of the capsule are encoded in what is historically called the “*viaB* locus,” which is composed of at least 10 genes (*tvIA-E* and *vexA-E*) at 93.9 centisomes on the chromosome (Virlogeux et al., 1995). Transcription of these genes is under the control of the *viaA* locus, which is now known to encode the two-component regulatory system RcsB and RcsC (Virlogeux et al., 1996).

The presence of the Vi capsule is determined by slide agglutination. If, on the basis of biochemical analysis, a strain is suspected to be serovar Typhi or Paratyphi C, then agglutination with Vi antisera is performed along with testing with the appropriate O group antisera. Note that the presence of the capsule can block antibody recognition of the O-antigen. Therefore, if a strain is positive for Vi antigen, the strain should be heated in boiling water for 15 min, cooled, and tested again. Upon heating, a serovar Typhi strain should be negative for Vi antigen and positive for O group D (see Difco, 1998).

### Nomenclature for Reporting Serotype

When designating the serotypes of *Salmonella* strains, the O antigen is given first, followed by the Vi (if present), and the H antigen(s), Phase 1 and then Phase 2. The major antigens are

separated by colons and the individual antigens by commas. For example, serovar Typhimurium is designated *Salmonella* 1, 4, [5], 12:i:1, 2. Note that antigens that result from phage lysogeny are underlined. Antigens in brackets are also variable, although not known to be mediated by viable phage. See Table 5 for further examples.

### Phage Typing and Other Methods of Subserotype Classification

*Salmonella* strains within a given serotype can be further differentiated using both classical and molecular techniques. Phage typing is one of the oldest methods for classification of *Salmonella* serotypes. Phage-typing systems exist for serotypes Typhi (Craigie and Yen, 1938), Typhimurium (Anderson, 1964; Anderson et al., 1977), Enteritidis (Ward et al., 1987), Dublin, Paratyphi A and Paratyphi B (Felix and Callow, 1943; Anderson, 1964), as well as other serotypes. These systems are based on the ability to plaque a defined set of phage, each of which has been propagated on a particular strain. Differential plaquing of the phage is dependent on a combination of factors (Schmieger, 1999). First is the presence of the appropriate phage receptor. Note that these are subtle differences between strains within a serotype and are likely due to modifications of the receptor by resident lysogenic phage. The Typhimurium typing phage, for example, generally use O-antigen as receptor (see O-antigen) and the Typhi phage use Vi antigen (see Vi antigen). Differences in phage sensitivity are subtler than can be detected using typing sera. Second are differences in host restriction-modification systems. Third are the presence of lysogenic phage or cryptic phage that can confer either immunity or superinfection exclusion systems. Note that there can also be recombination between the typing phage and resident phage yielding phage with new properties. The typing phage can also cause induction of resident phage (Schmieger, 1999). Because of the subtleties involved and the fact that the typing phage must be propagated precisely, if at all, phage typing is best done by particular reference laboratories.

Phage typing is a powerful and sensitive tool for epidemiology. For example, the emergence of particular strains such as serotype Typhimurium phage type DT104 can be monitored throughout the world (Cloeckert and Schwarz, 2001). Other techniques for subserotype classification include differentiating biochemical characteristics (bio-type), MLEE, or other molecular techniques (such as plasmid profiling, AFLP, or “variable number of tandem repeats”-based fingerprinting [VNTR]; Selander et al., 1996; Lindstedt et al., 2003; Soll et al., 2003). Indeed, VNTR apparently

Table 5. Serotypes for the most common *Salmonella* isolates (all subspecies I).

	Serovar	Somatic (O) antigen	Flagellar (H) antigen	
			Phase 1	Phase 2
Group A	Paratyphi A	<u>1</u> , 2, 12	a	—
Group B	Agona	4, 12	f, g, s	—
	Brandenburg	4, 12	l, v	e, n, z <sub>15</sub>
Group C <sub>1</sub>	Bredeney	<u>1</u> , 4, 12, <u>27</u>	l, v	1, 7
	Derby	<u>1</u> , 4, 12	f, g	—
	Heidelberg	<u>1</u> , 4, [5], 12	r	1, 2
	Paratyphi B	<u>1</u> , 4, [5], 12	b	1, 2
	Saintpaul	<u>1</u> , 4, [5], 12	e, h	1, 2
	Schwarzengrund	<u>1</u> , 4, 12, <u>27</u>	d	1, 7
	Typhimurium	<u>1</u> , 4, 5, 12	i	1, 2, [7]
	Braenderup	6, 7	e, h	e, n, z <sub>15</sub>
	Choleraesuis	6, 7	c	1, 5
	Infantis	6, 7, <u>14</u>	r	1, 5
	Livingstone	6, 7, <u>14</u>	d	l, w
	Mbandaka	6, 7	z <sub>10</sub>	e, n, z <sub>15</sub>
	Montevideo	6, 7	g, m, [p], s	[1, 2, 7]
	Oranienburg	6, 7	m, t	—
Group C <sub>2</sub>	Thompson	6, 7, <u>14</u>	[k]	[1, 5]
	Hadar	6, 8	z <sub>10</sub>	e, n, x
	Muenchen	6, 8	d	1, 2
Group C <sub>3</sub>	Newport	6, 8, <u>20</u>	e, h	1, 2
	Kentucky	8, <u>20</u>	i	z <sub>6</sub>
Group D <sub>1</sub>	Berta	<u>1</u> , 9, 12	[f], g, t	—
	Dublin	<u>1</u> , 9, 12, [Vi]	g, p	—
	Enteritidis	<u>1</u> , 9, 12	[f], g, m, [p], [t]	[1, 7]
	Javiana	<u>1</u> , 9, 12	l, z <sub>28</sub>	1, 5
	Typhi	9, 12, [Vi]	d	—
Group E <sub>1</sub>	Anatum	3, 10	e, h	1, 6
	Meleagridis	3, 10	e, h	l, w
Group E <sub>4</sub>	Senftenberg	1, 3, 19	g, [s], t	—
Group G	Mississippi	<u>1</u> , 13, 23	b	[1, 5]
	Poona	<u>1</u> , 13, 23	z	1, 6

Symbol: —, no antigenic variation.

<sup>a</sup>Antigens conferred by lysogenic phage are underlined. Antigens in brackets are also variable, although not known to be mediated by viable phage.

Adapted from Difco (1998).

detects subsets of phage type DT104 (Lindstedt et al., 2003).

## Preservation

Clinical or environmental samples should be used as soon as possible for isolation of *Salmonella* (Harrigan, 1998; Farmer, 1999). If this is not possible, food or environmental samples can be refrigerated (Harrigan, 1998); transport media is available for stool samples (Farmer, 1999). Pure stocks can be stored using several methods (Miller, 1992) including “stabs” in which a single colony is stabbed several times into sterile solid medium in a vial using a sterile loop. The vials are tightly sealed and maintained at room temperature. Although a time-honored tradition, this method is not recommended for long-term storage; the bacteria must continue to grow in the medium to maintain viability and evolution

is taking place (Edwards et al., 2001). More permanent storage is achieved by either lyophilization or freezing at  $-70^{\circ}\text{C}$  in  $\geq 10\%$  glycerol or  $10\%$  DMSO. Stocks will last decades under these conditions.

## Physiology

The *Salmonella*, in the family Enterobacteriaceae, are facultatively anaerobic Gram-negative, rod-shaped bacteria. Given the close relationship with *E. coli* and the many years devoted to its study, the overall metabolism of *Salmonella* is well understood. The cells are typically  $0.7\text{--}1.5\ \mu\text{m}$  by  $2\text{--}5\ \mu\text{m}$ . They will grow at  $7\text{--}48^{\circ}\text{C}$  with an optimum growth at  $37^{\circ}\text{C}$  (mesophile) and at pH 4.05–9.5 with an optimal growth at pH 6.5–7.5 (neutrophile). *Salmonella* grows optimally at a water activity of 0.995 (Cox, 1999).

*Salmonella* are chemoorganotrophs. They have both fermentative and oxidative metabolism. The primary route for metabolism of carbohydrates is the Embden-Meyerhof pathway (glycolysis). They ferment glucose to formate (with the production of gas), and to ethanol, acetate, or lactate. The electron transport chain is cytochrome-based with oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide (TMAO) as terminal electron acceptors. Enterics do not have a cytochrome *c* or cytochrome *c* oxidase in the transport chain and are classified, therefore, as “oxidase minus.” A more complete list of metabolic properties is given in Table 3. The specific metabolic characteristics that distinguish these organisms from other enterics are that *Salmonella* usually cannot metabolize lactose or sucrose, can hydrolyze 4-methylumbelliferyl caprylate (MUCAP), and produce hydrogen sulfide. Indeed, the genes for production of hydrogen sulfide can be used as *Salmonella*-specific molecular markers (Porwollik et al., 2002). Most serotypes of *Salmonella* are prototrophs; the exceptions are some of the host-adapted serotypes (Table 2). For examples, serotype Typhi strains are often cysteine and tryptophan auxotrophs, while serovar Pullorum strains require methionine, cysteine, thiamine and nicotinamide (at high temperature; K. Karberg, personal communication).

## Genetics

Genetic analysis in serovar Typhimurium is a high art form, and *Salmonella* rivals *E. coli* as far as our understanding of bacterial cell structure, biology and physiology is concerned (see <http://www.ecosal.org>{[www.ecosal.org](http://www.ecosal.org)}). Classic genetic tools, such as bacteriophage P22-mediated transduction (Maloy et al., 1996), combined with more modern techniques, such as Lambda Red-mediated recombination (Datsenko and Wanner, 2000; Ellermeier et al., 2002), provide the means to manipulate, at will, *Salmonella* strains for the study of any biological problem of interest.

The complete genome sequences of serovar Typhimurium strain LT2 (McClelland et al., 2001) and serovar Typhi strains Ty2 (Deng et al., 2003) and CT18 (Parkhill et al., 2001) are known (see {[www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)}). Representatives of additional *Salmonella* serovars are also being sequenced (see <http://www.salmonella.org>{[salmonella.org](http://www.salmonella.org)}). The LT2 genome is approximately 4857 kilobases with 4489 putative protein coding sequences. The DNA is 53 mol% G+C. In addition, the virulence plasmid, pSLT, is approximately 94 kilobases with 108 putative protein

coding regions. The pSLT plasmid is self transmissible (Ahmer et al., 1999a) and is indicative of virulence plasmids carried by most nontyphoid subsp. *enterica* strains (Guiney et al., 1995). The two serovar Typhi genomes are also approximately 4800 kilobases with approximately 4600 putative protein coding sequences. These two genomes are 98% identical. Although there is an obvious synteny in overall genomic organization, the two Typhi genomes differ from each other and from the LT2 genome by virtue of inversions between rRNA operons. Indeed, the host-restricted serovars are known to be prone to these chromosomal rearrangements for reasons that are unknown (Helm et al., 2003). The other striking difference is the large number (~200) of pseudogenes found in the Typhi strains. The loss of these genes presumably reflects the restricted host range, but there is no direct proof of this hypothesis. As has been found with virtually all bacterial genomic sequences, a significant number of genes are not found in the close relatives. For example, 29% of the LT2 genes are not found in *E. coli* K12 and 11% are not present in the Typhi genomes. These horizontally transferred genes are found throughout the chromosome on islands ranging from single genes to sets of genes (>50 kilobases). In several cases, these larger clusters are viable or cryptic phage genomes (see also Thomson et al., 2004).

What makes *Salmonella* unique from *E. coli* K12, of course, is pathogenesis. *Salmonella* serovars are capable of infecting a variety of different hosts and causing diseases ranging from a mild self-limiting gastroenteritis to a potentially lethal systemic infection (see Disease). Serovar Typhimurium normally causes a self-limiting gastroenteritis in humans but in a mouse is capable of causing a systemic infection that resembles human Typhoid fever caused by serovar Typhi. The mouse infection model and the sophisticated genetic techniques available for serovar Typhimurium have led to an extensive knowledge of host-pathogen interaction. Here, the focus is on the genetics of virulence in serovar Typhimurium in the mouse model. A brief overview of the infection cycle is provided, and the genes and gene products important at each stage are discussed. Reviews of particular aspects of *Salmonella* pathogenesis are referenced throughout.

## Overview of the *Salmonella* Infectious Cycle in Mice

*Salmonella* infection normally occurs via the ingestion of contaminated water or food products. *Salmonella* must survive numerous host



environments and defenses to produce a productive infection. The first of these barriers is the acidic condition encountered in the stomach. After passing through the stomach *Salmonella* colonizes the small intestine where it preferentially targets and invades the most distal Peyer's patch of the small intestine (Carter and Collins, 1974). Serovar Typhimurium can gain access to the underlying lymph tissue via M cells (phagocytic epithelial cells) and villous (nonphagocytic) epithelial cells of the small intestine using the *Salmonella* pathogenicity island 1 (SPI1) type three secretion system (TTSS). After gaining access to the underlying lymph tissue, serovar Typhimurium continues to replicate within the Peyer's patch tissue. Eventually serovar Typhimurium is disseminated to the target organs of the liver, spleen and bone marrow where the bacteria continue to replicate within macrophages eventually leading to septicemia and death of the host. Thus, *Salmonella* are facultative intracellular pathogens. Replication within macrophages requires a second TTSS, encoded on *Salmonella* pathogenicity island 2 (SPI2). As the number of organisms continues to increase, *Salmonella* can infect essentially all tissues of the body. Eventually, the host dies as a result of complications from septic shock and organ failure. During the infection *Salmonella* can be reintroduced into the small intestine via the gallbladder and disseminated into the environment. The ability of serovar Typhimurium to survive the various conditions and host defenses encountered during an infection requires a number of virulence factors, as well as the appropriate regulation of these virulence factors.

### Acid Tolerance Response

Infection by *Salmonella* is by the oral route as a result of ingesting contaminated food, water, or dairy products (Miller and Pegues, 2000). To produce a productive infection, *Salmonella* species must first survive the acidic conditions encountered in the stomach (Foster and Hall, 1990). Serovar Typhimurium can survive extremely low pH after growth in mildly acidic conditions (pH 4–5), a process termed “the acid tolerance response” (“ATR”; Audia et al., 2001). Serovar Typhimurium contains two distinct systems, the log phase ATR and stationary phase ATR. The log phase ATR induces expression of more than 50 proteins termed “acid shock proteins” (Hall and Foster, 1996). Several regulatory genes are required for the log phase ATR including *rpoS* (an alternative  $\sigma$  factor required for stationary phase gene expression), Fur (an iron sensing regulator of iron metabolism), and PhoPQ (a two-component regulatory system involved in regulation of other virulence genes; Lee et al.,

1995; Hall and Foster, 1996; Bearson et al., 1998; see PhoPQ). The second ATR system in serovar Typhimurium is an acid-induced RpoS-independent stationary phase ATR regulated by OmpR/EnvZ; Bang et al., 2000; Bang et al., 2002).

### Attachment and Colonization of the Small Intestine

Organisms that survive the gastric barrier colonize the small intestine. Numerous studies have shown that serovar Typhimurium preferentially attaches to and invades the most distal Peyer's patch of the small intestine (Carter and Collins, 1974; Jones et al., 1994; Wallis and Galyov, 2000). The apparent tropism of serovar Typhimurium for the M cells of the Peyer's patch as compared to the villous epithelial cells has led to the hypothesis that fimbriae mediate attachment of the bacteria to the M-cell surface. On the basis of the completed genome sequence, serovar Typhimurium potentially encodes 13 fimbrial operons (*agf* (*csg*), *fim*, *pef*, *lpf*, *bcf*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti* and *stj*; McClelland et al., 2001; Townsend et al., 2001; Edwards et al., 2002; Humphries et al., 2003). Production of 11 of these fimbriae has been detected, with expression being dependent on growth conditions (Humphries et al., 2003). Only a few of these fimbriae have been studied in any detail (reviewed in Humphries et al., 2001).

Serovar Typhimurium, like most Enterobacteriaceae, produces a type-1 fimbriae encoded by *fimAICDHF* that characteristically binds to mannose residues of host cells. A mutation in *fimA* significantly decreased the binding of serovar Typhimurium to mouse enterocytes (Thankavel et al., 1999). However, a *fim* mutation has only a minor effect on virulence in serovar Typhimurium (Lockman and Curtiss, 1992). Serovar Typhimurium SL1344 strains containing mutations in *fim* are unable to attach to or invade human epithelial cells in vitro but remain unaffected for invasion of cell lines from other host species. This suggests that the serovar Typhimurium type 1 fimbriae may mediate attachment to different hosts (Baumler et al., 1997a). Recent evidence suggests that there is a great deal of allelic variation within *fimH*, which encodes the adhesin (Boddicker et al., 2002).

Serovar Typhimurium also encodes fimbriae called “long polar fimbriae” (“Lpf”; Baumler and Heffron, 1995). The Lpf fimbriae mediate attachment of serovar Typhimurium to the M-cells of the Peyer's patch but not to villous epithelial cells of the small intestine (Baumler et al., 1996b). Mutations that disrupt Lpf increase the oral LD<sub>50</sub> in mice ca. fivefold and delay death compared to the wildtype by about 3 days

(Baumler et al., 1996b). The virulence plasmid of serovar Typhimurium, pSLT, contains a fimbrial operon termed “the plasmid-encoded fimbriae (*pef*) operon.” Mutations in the *pef* genes have been reported to decrease the ability of serovar Typhimurium to attach to the murine small intestine (Baumler et al., 1996a). Mutations in *pef* also increase the oral LD<sub>50</sub> ca. 2.5-fold (Baumler et al., 1996a).

Serovar Typhimurium contains a fourth fimbrial operon termed the “*agf* operon,” which produces thin aggregative fimbriae that are analogous to the curli fimbriae of *E. coli*. The Agf protein promotes bacterial autoaggregation and has been implicated in both biofilm formation and virulence (Romling et al., 2000). Mutations in *ompR* block transcription of the *agf* genes suggesting that OmpR is either directly or indirectly required for transcription of *agf* (Romling et al., 1998).

Recent evidence suggests that these operons may be functionally redundant. van der Velden determined that an *lpf pef* and *fim* triple mutant did not significantly decrease virulence, whereas a triple mutant of *lpf pef* and *agf* increased the oral LD<sub>50</sub> ca. 30-fold, and the quadruple mutant, *lpf pef agf* and *fim*, showed a similar increase in the oral LD<sub>50</sub> (Van der Velden et al., 1998). This strongly suggests that Lpf, Pef and Agf fimbriae are functionally able to compensate for one another in the BALB/c mouse model of infection (Van der Velden et al., 1998). Whether colonization of other hosts requires a different repertoire of fimbrial operons remains unclear, although the presence of such a large number of fimbrial operons suggests this may be the case. The contribution of the remaining nine fimbrial operons to virulence remains to be tested.

### Invasion of Epithelial Cells

A critical step in the infection process of *Salmonella* species is the ability to invade the intestinal epithelial barrier (Darwin and Miller, 1999b). Serovar Typhimurium may be capable of breaching the epithelium in more than one way. Serovar Typhimurium invades normally nonphagocytic intestinal epithelial cells using a type three secretion system (TTSS) encoded by *Salmonella* pathogenicity island 1 (SPI1; Darwin and Miller, 1999b; Galan, 1999; Lostroh and Lee, 2001b). The SPI1 TTSS forms a needle-like structure capable of injecting so-called “effector proteins” directly into the cytosol of host cells (Kubori et al., 1998; Kubori et al., 2000; Kimbrough and Miller, 2000; Kimbrough and Miller, 2002). SPI1 effectors cause a rearrangement of the eukaryotic cell cytoskeleton, facilitating bacterial engulfment and invasion of the intestinal epithelium. Injection of these proteins also induces a

series of proinflammatory cytokines that trigger mucosal inflammation and infiltration of neutrophils (Wallis and Galyov, 2000). This inflammatory response is thought to contribute to gastroenteritis symptoms associated with *Salmonella* infection (Wallis and Galyov, 2000; see Gastroenteritis).

The SPI1 locus comprises approximately 35 genes that encode components of secretion apparatus, several effector proteins, transcriptional regulators of genes encoded both within SPI1 and elsewhere in the chromosome, and chaperones required for assembly of the apparatus or secretion of effectors (Lostroh and Lee, 2001b).

### The SPI1 TTSS Apparatus

Approximately 17 proteins, homologs of which are found in essentially all type III systems (Hueck, 1998), comprise the secretion apparatus and form a “needle complex” that has a cytoplasmic “export apparatus,” inner membrane components, outer membrane components, and the needle, which extends out from the cell (Kimbrough and Miller, 2002). Presumably, target proteins are secreted directly from the cytoplasm through this needle. Exported proteins include SipB, SipC and SipD, which are proposed to act as a “translocon” that integrates into the eukaryotic cell membrane as a prerequisite for delivery of other effector proteins (Darwin and Miller, 1999b; Lostroh and Lee, 2001b). Interaction of the needle with the translocon is assumed to complete the apparatus and allow direct injection of effector proteins.

### SPI1 Effector Proteins

The most striking result of SPI1 TTSS action is an actin cytoskeletal rearrangement that leads to bacterial engulfment and invasion. The SPI1 TTSS injects a number of effector proteins responsible for this and other physiological responses. These include the SPI1 encoded effectors SipA (SspA), AvrA, and SptP; the SPI1 encoded translocon proteins SipB, SipC and SipD (which apparently also have effector function); and effectors that are encoded on pathogenicity islands or bacteriophage elsewhere in the chromosome, SopA, SopB (SigD), SopD, SopE1, SopE2, SspH1 and SlrP. The biochemical functions of the various effectors and their roles in *Salmonella* pathogenesis are just beginning to be understood.

Individual mutations in *sopE1*, *sopE2* or *sopB* result in only minor invasion defects, whereas a strain containing mutations in all three genes has a severe invasion defect (Zhou and Galan, 2001a), suggesting that these effectors are functionally redundant. The *sopE1* and *sopE2* genes,

encoded by the SopE phage and within a *Salmonella* specific region near *proQ*, respectively, are paralogs that promote actin rearrangement by acting as GDP/GTP exchange factors for two members of the Rho family of GTPases, Rac1 and Cdc42 (Bakshi et al., 2000; Friebel and Hardt, 2000; Stender et al., 2000; Friebel et al., 2001). The activation of Rac1 and Cdc42 promotes large-scale actin rearrangement resulting in the characteristic membrane ruffling and engulfment of the bacteria (Zhou et al., 2001b), as well as triggering a signal transduction cascade that leads to the activation of NF- $\kappa$ B (Hobbie et al., 1997). SopB acts as an inositol phosphatase and produces a molecule that acts as an indirect activator of Cdc42 (Norris et al., 1998).

SipA and SipC are actin-binding proteins that promote bacterial mediated endocytosis. The binding of SipA to actin is believed to decrease the critical concentration required for actin polymerization by inhibiting actin depolymerization and binding of T-plastin (Zhou et al., 1999a; Zhou et al., 1999b; Higashide et al., 2002). SipC is a two-domain protein that can act to nucleate and bundle actin (Hayward and Koronakis, 1999). The N-terminal portion of SipC binds F-actin, and the C-terminus is required for actin nucleation (Hayward and Koronakis, 1999). The relevance of these activities in vivo has yet to be determined because a *sipC* mutation completely blocks translocation of all the effectors, as it is a member of the translocon (Collazo and Galan, 1997).

SptP is a GTPase-activating protein for both CDC42 and Rac1 that promotes actin depolymerization, opposing the activating functions of SopE, SopE2 and SopB (Fu and Galan, 1999). During invasion, serovar Typhimurium causes large-scale actin rearrangement. After internalization of serovar Typhimurium, the cells are able to return to a normal cellular architecture (Galan and Zhou, 2000). SptP has been suggested to play a role in this recovery because of its GTPase activating protein (GAP) activity (Galan and Zhou, 2000). SspH1 (Haraga and Miller, 2003) and AvrA (Collier-Hyams et al., 2002) also attenuate the role of other effectors by downregulating NF- $\kappa$ B activation. A null mutant of *sptP* decreases invasion ca. twofold (Darwin and Miller, 1999b). Loss of SspH1 alone does not confer a significant defect in a calf model (Miao et al., 1999), nor does loss of AvrA confer an obvious phenotype (Hardt and Galan, 1997; Zhang et al., 2002).

SipB, also a member of the translocon, has been shown to interact with caspase-1 to induce necrosis in macrophages (see Peyer's Patch Survival). The biochemical activities of SlrP, SopA and SopD are unknown. Phenotypically, mutations in *sopA* or *sopD* (as well as *sipA*, *sopB* and

*sopE2*) significantly reduce fluid accumulation in a calf model of *Salmonella*-induced diarrhea (Zhang et al., 2002). Mutants lacking SlrP are attenuated in a mouse model after oral infection but are fully virulent in the calf model (Tsolis et al., 1999).

### SPI1 TTSS Chaperones

Chaperones are often required for the secretion and translocation of effector proteins in TTSS. Serovar Typhimurium contains three known SPI1 related chaperones: InvB acts as chaperone for SipA (Bronstein et al., 2000), SopA (Ehrbar et al., 2004), SopE1 (Lee and Galan, 2003) and SopE2 (Ehrbar et al., 2003); SicA acts on SipB and SipC (Tucker and Galan, 2000); and SicP is the chaperone of SptP (Stebbins and Galan, 2001; Lee and Galan, 2004). The presence of SicP and InvB increases stability of their respective effectors SptP and SipA without affecting the transcriptional expression of either *sptP* or *sipA* (Bronstein et al., 2000; Stebbins and Galan, 2001). The case of SicA is more complicated as mutants of *sicA* decrease expression of the *sic/sip* operon at the transcriptional level (Tucker and Galan, 2000; see SPI1 Encoded Regulators). InvE also seems to play a role in secretion by interacting with a chaperone-effector complex (Kubori and Galan, 2002).

### SPI1 Encoded Regulators of the SPI1 TTSS

The expression of the SPI1 TTSS is controlled in response to a specific combination of environmental signals that presumably act as a cue that the bacteria are in the appropriate anatomic location (Bajaj et al., 1996; Schechter et al., 1999; Slauch, 2000). In the laboratory, the system is active when cells are grown under "SPI1 inducing conditions"-high osmolarity and low oxygen. Regulation is mediated primarily via control of the level of HilA, a member of the OmpR/ToxT family of transcriptional regulators encoded on SPI1 (Lee et al., 1992; Bajaj et al., 1995; Lostroh and Lee, 2001a). HilA directly activates expression of the *prg/org* and *inv/spa* operons in SPI1 by binding just upstream of the -35 sequences of  $P_{prgH}$  and  $P_{invF}$  (Lostroh and Lee, 2001a). Activation of  $P_{invF}$  increases production of InvF, a member of the AraC family of transcriptional regulators (Kaniga et al., 1994). InvF then induces expression of effector proteins encoded both within and outside of SPI1, including *sicA* (SPI1), *sopE* (SopE phage in strain SL1344) and *sopB* (SPI5; Lostroh and Lee, 2001a).

Activation of promoters by InvF requires SicA, a TTSS chaperone (Darwin and Miller, 1999a; Eichelberg and Galan, 1999a; Darwin and Miller, 2000), which has been suggested to stabi-

lize a complex between InvF, RNA polymerase, and DNA (Darwin and Miller, 2001). This raises the intriguing possibility that once the secretion apparatus is assembled, the effector proteins are secreted, resulting in free SicA. The free SicA can then form a complex with InvF to activate expression of numerous effector proteins. This constitutes a negative feedback loop that couples expression of effector proteins to assembly of the TTSS, analogous to the FliA/FlgM controlled expression of the class three flagellar genes (Chilcott and Hughes, 2000). As of yet there is no proof of this model, but it remains an attractive hypothesis.

Another gene located on SPI1, *sprB*, appears to lie in an operon with *hilC* and has been hypothesized to be a regulator because the predicted protein sequence contains a LuxR-like helix-turn-helix motif (Eichelberg et al., 1999b). To date neither overexpression of *sprB* nor *sprB* null mutations affect expression of any of the SPI1 encoded genes tested (Eichelberg et al., 1999b). Still unknown is whether SprB regulates genes located outside of SPI1, or SprB can regulate the expression of invasion genes under different conditions than those tested.

### Direct Regulators of *hilA*

Expression of *hilA* is the apparent integration point for a variety of signals that control the expression of the invasion genes. Two SPI1 encoded proteins, HilC (SirC or SprA) and HilD, both members of the AraC/XylS family of transcriptional regulators, induce expression of *hilA* (Eichelberg et al., 1999b; Rakeman et al., 1999; Schechter et al., 1999). Loss of HilD decreases expression of *hilA* ca. tenfold under SPI1 inducing conditions, whereas a mutation of *hilC* reduces expression of *hilA* ca. twofold (Lucas and Lee, 2001). HilC and HilD bind to the *hilA* promoter region, and binding is believed to induce the expression of *hilA* (Schechter et al., 1999; Schechter and Lee, 2001; Olekhnovich and Kadner, 2002). HilC and HilD also induce expression of the *inv/spa* operon, and hence *invF*, independent of HilA (Eichelberg et al., 1999b; Rakeman et al., 1999; Akbar et al., 2003).

We have recently identified two regulatory proteins, termed RtsA and RtsB, which are encoded in an operon located on a 15-kb island inserted near the tRNA<sup>PheU</sup> gene (Ellermeier and Slauch, 2003). RtsA belongs to the AraC/XylS family of regulators and RtsB is a helix-turn-helix DNA binding protein. RtsA, like its paralogs, HilC and HilD, increases expression of *hilA* by direct binding of RtsA to the *hilA* promoter. RtsA also induces expression of *hilD*, *hilC* and the *invF* operon. In addition to activating SPI1-related genes via induction of InvF, RtsA is capa-

ble of activating, independently of HilA or InvF, at least one effector gene, *slrP* (Ellermeier and Slauch, 2003), and *dsbA*, which encodes a periplasmic disulfide bond isomerase required for function of the SPI1 TTSS (Ellermeier and Slauch, 2004). RtsB represses expression of the flagellar genes by binding to the *flhDC* promoter region. Repressing production of the positive activators FlhDC decreases expression of the entire flagellar regulon. Thus RtsA and RtsB appear to coordinate induction of invasion and repression of motility in the small intestine.

We propose that RtsA, HilC and HilD act as a feed-forward loop to activate *hilA* and hence the SPI1 TTSS (C. D. Ellermeier et al., unpublished hypothesis). These three homologous AraC-like regulators are seemingly redundant, in that they can each independently activate *hilA*. However, they each can also independently activate themselves and each other, e.g., RtsA activates *rtsAB*, *hilC*, *hilD*, etc. Thus, activation of any of the three should activate the entire system. Indeed, we have shown that at least two of these regulators must be present for significant induction of SPI1 either in the laboratory or during infection (C. D. Ellermeier et al., unpublished observation). AraC-like regulators often interact with a small molecule, like arabinose, to activate transcription. Possibly, RtsA, HilC, and HilD each respond to an independent signal to activate SPI1, but what these signals are is not clear.

### Global Regulators of the SPI1 TTSS

Genetic studies have identified a wide array of regulatory proteins encoded outside of SPI1 that impinge on expression of *hilA*. This includes several two-component regulatory systems (for a review, see Hoch and Silhavy [1995] and Bijlsma and Groisman [2003]). PhoPQ, a significant regulator of virulence and physiology (Groisman, 2001), represses expression of the SPI1 TTSS (Behlau and Miller, 1993; Pegues et al., 1995) in response to low magnesium concentrations (Chamnongpol et al., 2003). The PhoBR two-component regulatory system also represses expression of *hilA* (Lucas et al., 2000; Lucas and Lee, 2001), presumably in response to its normal signal, low extracellular phosphate ion concentration. Mutations that disrupt the osmoregulatory two-component system OmpR/EnvZ reduce expression of *hilA* but do not alter the osmoregulation of *hilA* (Lucas and Lee, 2001). SirA (the response regulator)/BarA (the sensor kinase) is a two-component regulatory system required for maximal expression of *hilA*; *sirA* mutations decrease expression of *hilA* ca. tenfold (Johnston et al., 1996; Ahmer et al., 1999b; Rakeman et al., 1999; Lucas and Lee, 2001). BarA/SirA have

recently been implicated in controlling SPI1 gene expression in response to bile (Prouty and Gunn, 2000).

In *E. coli* the SirA/BarA homologues, UvrY/BarA, are involved in a regulatory loop with *csrAB* (Suzuki et al., 2002), which encode a protein-RNA pair that function as both positive and negative regulators of gene expression. CsrA is a 61-amino acid protein that can directly bind mRNA transcripts tagging them for degradation (Romeo, 1998). The RNA partner *csrB* is a 360-nucleotide RNA that antagonizes the activity of CsrA by binding 18 CsrA molecules (Romeo, 1998). In the absence of *csrB*, CsrA is free to bind mRNA and tag these transcripts for degradation (Romeo, 1998). Either a null mutation in *csrB* or increased expression of *csrA*<sup>+</sup> decreases expression of *hilA* (Altier et al., 2000a; Altier et al., 2000b). Also, a *csrB* null mutant was observed to decrease the steady state levels of *hilC* and *hilD* mRNA (Altier et al., 2000a). Still unclear is whether this decrease is a direct effect of CsrA binding to the *hilC* and *hilD* transcripts and targeting them for degradation. A mutation in *csrA* also decreases expression of the invasion genes suggesting that CsrA can act as both a positive and negative regulator of *hilA* expression (Altier et al., 2000a).

Several other regulatory systems have been implicated in SPI1 regulation. A mutation in the gene *hilE* increases expression of *hilA* under SPI1 inducing and repressing conditions (Fahlen et al., 2000). HilE represses expression of *hilA* by interacting with HilD (Baxter and Jones, 2003). Indeed, recent data suggests that several, if not all, of the two-component systems feed into the HilD, HilC, RtsA regulatory network through HilD, perhaps via HilE (Baxter and Jones, 2003; C. D. Ellermeier et al., unpublished data).

Mutations in *sirB*, *lon*, *hupB*, *hha* and *fis* alter expression of *hilA* under both *hilA* inducing and repressing conditions (Johnston et al., 1996; Fahlen et al., 2000; Fahlen et al., 2001; Wilson et al., 2001; Takaya et al., 2002). Hha and Lon decrease expression of *hilA* while Fis, IHF, and SirB increase expression of *hilA*. Indeed, Lon has recently been implicated in downregulating HilA after invasion has taken place (Boddicker and Jones, 2004). A previously unidentified PhoP-activated gene (*pag*) has also been shown to negatively regulate *hilA* expression (Fahlen et al., 2000). With the exception of Hha, which binds to the *hilA* promoter region, it remains mechanistically unclear how these gene products control expression of *hilA* (Fahlen et al., 2001). Several studies have identified the class 3 flagellar protein FliZ as an activator of *hilA* expression (Eichelberg and Galan, 2000; Lucas et al., 2000; Iyoda et al., 2001), although still unclear is how

FliZ activates expression of *hilA*. A number of mutations in the SPI2 TTS apparatus repress expression of *hilA* (Deiwick et al., 1998). The molecular mechanism and physiological relevance of these mutations on expression of *hilA* also remains unclear.

## Gastroenteritis

The mechanism by which serovar Typhimurium causes gastroenteritis is not completely clear, but the current model suggests it is caused by inflammation, increased fluid accumulation within the small intestine, and polymorphonuclear leukocyte (PMN) influx into the small intestine (McCormick et al., 1995; Darwin and Miller, 1999b; Zhang et al., 2003). Most virulence studies on serovar Typhimurium use a mouse model of infection. One caveat is that a serovar Typhimurium infection of a mouse (unlike that of a human) does not result in gastroenteritis or diarrhea. Therefore, several groups have used a calf model of infection to study how serovar Typhimurium causes gastroenteritis (Zhang et al., 2003). In calves, serovar Typhimurium causes diarrhea, inflammation of Peyer's patches, fluid accumulation in the small intestine, and PMN influx into the small intestine (Zhang et al., 2003). Clearly, mutants of the SPI1 TTSS are severely decreased in the ability to cause gastroenteritis in calves (Tsolis et al., 2000; Zhang et al., 2002), and specific SPI1 effector proteins have been implicated (Zhang et al., 2002; see SPI1 Effectors).

Various tissue culture systems have also been used to model the interaction of *Salmonella* with the intestinal epithelium, leading to a complex picture of potential disease mechanisms (Hurley and McCormick, 2003). Serovar Typhimurium strains are capable of inducing polarized epithelial cell lines to secrete the proinflammatory cytokine, IL-8, primarily from the basilar side (McCormick et al., 1993). Secretion of IL-8 may form a gradient, which recruits PMNs to the lamina propria. Serovar Typhimurium induces PMN migration across the epithelial barrier by inducing the secretion of a pathogen-elicited epithelial chemoattractant (PEEC) on the apical side (McCormick et al., 1998). Other chemokines have also been detected (Hurley and McCormick, 2003). Interestingly neither *S. typhi* nor *S. paratyphi* elicit secretion of PEEC, nor do they promote transepithelial migration of PMNs in vitro, consistent with the limited diarrhea often observed in infections with these organisms (McCormick et al., 1995).

In nonpolarized epithelial cells, the injected SPI1 effectors SopE1, SopE2, and SopB (see Effectors) activate CDC42 and Rac1 leading to

not only cytoskeletal rearrangements, but also activation of NF- $\kappa$ B and IL-8 production (Chen et al., 1996a; Hardt et al., 1998). In polarized cells, however, the target of these effectors is apparently Rac1 at the apical surface, and interaction stimulates cytoskeletal rearrangement but not activation of the signal cascade (Hobert et al., 2002). Thus, the effectors do not directly activate IL-8 production. However, effector-mediated CDC42 and Rac1 activation are required for IL-8 release at the basolateral surface of the polarized cells (Hobert et al., 2002).

NF- $\kappa$ B and hence IL-8 are apparently stimulated by bacterial flagellin activating Toll-like receptor 5 (TLR5), which is only expressed on the basolateral surface of polarized cells (Gewirtz et al., 2001a; Hayashi et al., 2001). Serovar Typhimurium strains lacking flagellin such as a *fljC fljB* double mutant are severely decreased in their ability to elicit IL-8 secretion (Gewirtz et al., 2001b). Purified FliC added to the apical surface of polarized epithelial cells does not induce secretion of IL-8, while addition of FliC to the basolateral surface does (Gewirtz et al., 2001b). This suggests that epithelial cells are able to sense and respond to invasive bacteria by sensing the presence of the flagellin on the basolateral surface without responding to commensal organisms normally present on the apical surface.

PEEC is secreted apically by the polarized epithelial cells and directs PMN migration across the epithelial monolayer (McCormick et al., 1998). A *sipA* mutant (see SPI1 Effectors) was shown to be unable to elicit PEEC secretion (Lee et al., 2000b). Interestingly, translocation of SipA is not required because the addition of purified SipA is sufficient to elicit transepithelial migration of PMNs (Lee et al., 2000b). Mutation of *sipA* does not affect *Salmonella* induced secretion of IL-8. These data suggest that SPI1 effectors and flagellin act in concert, but at distinct steps, to initiate an inflammatory response at the intestinal mucosa.

### SPI1-Independent Mechanisms of Breaching the Epithelial Barrier

Although serovar Typhimurium utilizes the SPI1 TTSS to induce invasion of nonphagocytic epithelial cells, it is not absolutely required to produce a systemic infection, suggesting that multiple pathways exist by which serovar Typhimurium can cross the epithelial barrier (Jones et al., 1994; Penheiter et al., 1997). Vazquez-Torres et al. (1999) reported that serovar Typhimurium could be transported across the epithelial barrier within CD18+ cells. They utilized a serovar Typhimurium strain containing an *invG* mutation, which blocks SPI1 type III secretion and invasion, and an *lpf* mutation, which should block

targeting of *Salmonella* to M cells (Baumler et al., 1996b; Baumler et al., 1997b). Whereas these mutations clearly reduce virulence, the mutant bacteria could be found in the bloodstream within 15 min after an oral infection (Vazquez-Torres et al., 1999). The authors showed that CD18 knock-out mice, when compared to wild-type mice, had decreased numbers of serovar Typhimurium in their spleen after an oral infection with the mutant bacteria, whereas after intraperitoneal (IP) infection the CD18 knock-out mice actually contained increased numbers of serovar Typhimurium in the spleen (Vazquez-Torres et al., 1999). This suggests that CD18 cells (neutrophils?) are responsible for the transport of these mutant serovar Typhimurium from the small intestine to the spleen. That CD18+ cells actually transport serovar Typhimurium across the epithelial cells has been suggested but not demonstrated (Vazquez-Torres et al., 1999). Strains containing *invA lpfC* double mutants can still be phagocytosed by M cells and passed to the underlying lymph tissue. Therefore, CD18+ cells may not need to cross the membrane to transport serovar Typhimurium. Another model is that the M cells phagocytize serovar Typhimurium and pass them to the CD18+ cells in the underlying lymph tissue of the Peyer's Patch. The CD18+ cells can then disseminate serovar Typhimurium to the target organs. Interestingly the authors showed that SPI1 mutants were unable to elicit an IgA mucosal immune response but were fully capable of eliciting a systemic IgG response (Vazquez-Torres et al., 1999). This does suggest that the Peyer's patches were bypassed in this situation.

### Peyer's Patch Survival

In mice, serovar Typhimurium preferentially invade and colonize the distal ileal Peyer's patches (Carter and Collins, 1974; Jones et al., 1994; Wallis and Galyov, 2000), specialized lymphoid tissue designed to sample intestinal antigens and lead to an immune response that preferentially involves the production of IgA. Invasion results in destruction of the M cell and inflammation of the Peyer's patch (Jones et al., 1994). Recent data suggest that *Salmonella* is primarily found within Peyer's patch dendritic cells (Hopkins and Kraehenbuhl, 1997; Hopkins et al., 2000). Survival of serovar Typhimurium within dendritic cells does not appear to require the same virulence factors required for macrophage survival (Niedergang et al., 2000; see Survival within Macrophages). Other data suggest that *Salmonella* induction of necrosis in macrophages (Brennan and Cookson, 2000; Watson et al., 2000), mediated by the SPI1 effector protein SipB interaction with caspase-1, is critical for

growth in Peyer's patches (Chen et al., 1996b; Monack et al., 1996; Monack et al., 2000; Hersh et al., 1999; Lundberg et al., 1999). Caspase-1 or ICE (interleukin-1 $\beta$ -converting enzyme) is required for processing of IL-1 $\beta$  but is also capable of inducing necrosis, an inflammatory event. In caspase-1 deficient mice, bacteria were not recovered in significant numbers from the Peyer's patches or any deeper tissue (Monack et al., 2000). In ligated-loop experiments, the bacteria traverse the M cells of caspase-1 deficient mice. Also, there is no apparent difference in infection of wildtype versus caspase-1 knockout mice when the bacteria are injected intraperitoneally. Thus, caspase-1 is required for serovar Typhimurium to propagate in the Peyer's patch. We have previously shown that *gipA*, encoded on the Gifsy-1 prophage, is specifically required for growth in the Peyer's patch (Stanley et al., 2000). These results suggest that the bacteria sense and respond to the particular environment of the Peyer's patch and that a subset of virulence factors will be required to grow in this tissue.

The bacteria continue to propagate in the Peyer's patch. Whether this growth is extracellular or intracellular is not clear. Also, if growth of serovar Typhimurium is intracellular, what eukaryotic cell types are involved is unknown. Serovar Typhimurium could potentially interact with any cell type present in the Peyer's patch. In addition to dendritic cells and macrophages, neutrophils are clearly present in the Peyer's patch after initial infection (Monack et al., 2000). This is consistent with the fact that caspase-1-mediated necrosis should initiate inflammation. Also known is that serovar Typhimurium can invade B and T cells in tissue culture (Verjans et al., 1994). Presumably, the SPI1 type III secretion system is no longer induced in the Peyer's patch environment and subsequent intracellular growth does not require this system. Serovar Typhimurium possesses a second type III secretion system encoded on SPI2 (see below). This system is induced during and is required for intracellular growth in macrophages in vitro (Cirillo et al., 1998). Serovar Typhimurium strains mutant in SPI2 propagate in the Peyer's patch, although the numbers of bacteria are slightly reduced compared to the wildtype strain. These SPI2 mutants are incapable of surviving beyond the Peyer's patch tissue (Cirillo et al., 1998).

### Survival within Macrophages

After propagating in the Peyer's patch, serovar Typhimurium is spread systemically to target organs, particularly the spleen, liver, and bone marrow (Collins, 1970; Carter and Collins, 1974). In mice, the bacteria continue to increase in

numbers, eventually causing death. An important aspect of systemic growth is the ability of serovar Typhimurium to survive in macrophages (Collins, 1970; Carroll et al., 1979). The role of neutrophils in *Salmonella* infection is more controversial (Cheminay et al., 2004). Several lines of evidence support the macrophage as the primary site of replication during systemic infections (Richter-Dahlfors et al., 1997; Salcedo et al., 2001), and serovar Typhimurium mutants that cannot grow in tissue culture macrophages are avirulent (Fields et al., 1986; Buchmeier and Heffron, 1989). During growth within macrophages, serovar Typhimurium replicates within a specialized *Salmonella* containing vacuole (SCV; Holden, 2002). Long tubular structures called "*Salmonella* induced filaments" ("Sifs"; Garcia-del Portillo et al., 1993) are also evident. These filaments are rich in lysosomal glycoproteins, extend from the SCV, and require microtubule formation (Stein et al., 1996). The SCV and Sifs are actively maintained by serovar Typhimurium and their formation is dependent on several virulence factors.

Phagocytes normally kill engulfed bacteria using a battery of toxic substances. Phagosomes fuse with lysosomes, which contain hydrolytic degradative enzymes, antimicrobial peptides, and NADPH-dependent oxidase, which produces superoxide (Clark, 1999). Activated macrophages also produce nitric oxide, generated from arginine and oxygen by the inducible nitric oxide synthase (iNOS; Miller and Britigan, 1997). The nitric oxide can react with superoxide to produce highly toxic peroxynitrite (Miller and Britigan, 1997; Storz and Imlay, 1999). Other reactive oxygen species (ROS) and reactive nitrogen species (RNS) are also generated (Storz and Imlay, 1999). Serovar Typhimurium apparently has the ability to both prevent the induction or delivery of these enzymes to the SCV and survive these insults once induced (Ernst et al., 1999; Uchiya et al., 1999; Vazquez-Torres et al., 2000; Gallois et al., 2001).

Generation of superoxide by macrophages is dependent upon NADPH-dependent oxidase (phox). Normally serovar Typhimurium blocks delivery of the NADPH-dependent oxidase by blocking phagosome lysosome fusion in a SPI2 TTSS-dependent manner (Gallois et al., 2001; Vazquez-Torres and Fang, 2001; see TTSS). Thus one mechanism by which serovar Typhimurium avoids death by superoxide is by blocking the delivery of the host enzyme responsible for its production.

### The SPI2 TTSS Apparatus

Serovar Typhimurium possesses a second TTSS encoded on SPI2 (Hensel, 2000). In addition to

encoding proteins related to tetrathionate reductase and several genes of unknown function, the 40-kb locus encodes 31 ORFs organized into two operons that encode the secretion system apparatus (*ssa* genes), one operon encoding effector and chaperone proteins (*sse* and *ssc* genes, respectively) and an operon encoding the two-component regulatory system SsrAB (*ssr* for secretion system regulator; Hensel, 2000). SPI2 mutants are incapable of surviving beyond the Peyer's patch (Cirillo et al., 1998). This system is induced during intracellular growth in macrophages (Cirillo et al., 1998; Pfeifer et al., 1999), and apparently injects its effector proteins into the macrophage cytoplasm from within the phagosome (Hensel, 2000). The SPI2 apparatus structure has not been studied in detail and is, rather, deduced from the structure of homologous systems. The three "effector" proteins SseBCD function as the translocon, which is embedded in the phagosome membrane and required for translocation of other effectors.

### SPI2 Effector Proteins and Chaperones

There are 13 known SPI2 effector proteins in addition to the translocon proteins SseBCD. These include the SPI2-encoded proteins SpiC, SseF and SseG. Effectors SifA, SifB, SlrP, SopD2, PipB and PipB2 are encoded on pathogenicity islands or islets, while SseI, SseJ, SspH1 and SspH2 are encoded on bacteriophage. SlrP and SspH1 are translocated by both the SPI1 and SPI2 TTSS. The specific functions of these various effector proteins are only beginning to be understood (Waterman and Holden, 2003). Approximately 11 additional genes have been identified as being regulated by SsrB, but these have not been shown to be translocated proteins (Worley et al., 2000).

The putative effector protein SpiC has been reported to block vesicular trafficking in *Salmonella* infected macrophages, thereby preventing the formation of mature phagolysosomes (Uchiya et al., 1999). Indeed, ectopic production of SpiC within a macrophage was capable of blocking phagosome-lysosome fusion (Uchiya et al., 1999). SpiC apparently interacts with the macrophage protein TassC, which has a suggested role in vesicular trafficking (Lee et al., 2002). However, SpiC is required for secretion of the translocon components SseBCD, and thus the translocation of all effectors (Freeman et al., 2002; Yu et al., 2002). SpiC is not essential for secretion of SPI2 effector proteins into the medium (Freeman et al., 2002; Yu et al., 2002). Thus, SpiC might have multiple roles.

The *sifA* gene was originally identified as essential for the formation of tubular membrane structures (Sifs) in *Salmonella*-infected epithelial

cells (Stein et al., 1996). These structures also occur in macrophages as extensions of the SCV. SifA production is dependent on SPI2 regulatory genes, and *sifA* mutants are defective for replication in macrophages and are attenuated in vivo (Beuzon et al., 2000; Guy et al., 2000). SseJ (Kuhle and Hensel, 2002; Ruiz-Albert et al., 2002; Freeman et al., 2003), SseF and SseG (Guy et al., 2000; Kuhle and Hensel, 2002) have also been implicated in Sif formation.

### Regulation of the SPI2 TTSS

The expression of the SPI2 locus is induced during growth within macrophages (Valdivia and Falkow, 1997). Induction of SPI2 encoded genes is dependent upon a SPI2-encoded two-component regulatory system SsrAB (Cirillo et al., 1998). SsrA is a sensor kinase and SsrB is a response regulator. Induction of the SPI2 TTSS occurs at low pH (<6) and is independent of magnesium concentration and the PhoPQ two-component regulatory system (Deiwick et al., 1999; Miao et al., 2002). Expression of *ssrAB* itself is induced during growth within macrophages (Lee et al., 2000a). The layout of the *ssrA* and *ssrB* genes is unusual in that they are separately transcribed; SsrB activates its own promoter but not that of *ssrA* (Feng et al., 2003). Both transcripts are activated by the two-component regulatory system OmpR/EnvZ. Phosphorylated OmpR binds both the *ssrA* and *ssrB* promoter regions (Lee et al., 2000a; Feng et al., 2003).

### The PhoPQ Regulon

The PhoPQ two-component system controls the expression of greater than 40 genes termed "pags" ("PhoP activated genes," which include the *phoPQ* operon) and "prgs" ("PhoP repressed genes," which include the SPI1 TTSS; Groisman, 2001). PhoQ, the sensor kinase, appears to autophosphorylate in response to low  $Mg^{+2}$  and  $Ca^{+2}$  concentrations (Soncini et al., 1996). PhoQ then phosphorylates PhoP, which activates or represses gene expression (Groisman, 2001). Null mutations in either *phoP* or *phoQ* increase the LD<sub>50</sub> ca. 5 logs after IP infection (Miller et al., 1989). Constitutively active *pho* mutants also decrease virulence after both oral and IP infections (Miller and Mekalanos, 1990), suggesting that the appropriate regulation of virulence genes is important for survival.

Groisman (2001) suggests that the PhoPQ regulon is responsible for several overlapping functions: adaptation to low  $Mg^{+2}$ , regulation of virulence, including regulation of genes acquired by horizontal gene transfer, and modification of the cell envelope. PhoPQ activate two of the three genes or operons that encode  $Mg^{+2}$  trans-



porters: *mgtA* and *mgtCB*. MgtA and MgtB are each members of the P-type ATPase family of ion transporters (Moncrief and Maguire, 1999). MgtC, encoded with MgtB is essential for virulence but is apparently not involved in  $Mg^{+2}$  transport (Blanc-Potard and Groisman, 1997; Moncrief and Maguire, 1998). Other PhoP-activated genes with known biochemical functions include *pagP* (which encodes an outer membrane protein responsible for incorporating palmitate into lipid A, imparting resistance to the antimicrobial peptide C18G; Bishop et al., 2000); *pgtE* (which encodes an outer membrane protease that can degrade antimicrobial peptides; Guina et al., 2000); and *pcgL* (which encodes a D-Ala-D-Ala dipeptidase; Mouslim et al., 2002). PhoP represses expression of the SPI1 secretion system (see SPI1 TTSS) during intracellular growth. The molecular functions of most of the PhoP-regulated genes are unknown.

PhoPQ activate expression of the *pmrD* gene, whose product posttranscriptionally activates the PmrAB two-component regulatory system (Gunn and Miller, 1996; Groisman, 2001). PmrAB then regulates expression of a number of genes that are involved in resistance to antimicrobial peptides, such as polymyxin B (Gunn et al., 2000). One resistance mechanism is modification of LPS. PmrA induces expression of *pmrE* and *pmrHFIJKLM*, which encode proteins that synthesize a modified lipid A by adding 4-aminoarabinose (Gunn et al., 1998). This results in a more positively charged LPS, which is thought to inhibit the actions of negatively charged antimicrobial peptides and also compensates for loss of  $Mg^{+2}$ , required to counteract the negative charges on the LPS (Groisman et al., 1997; Groisman et al., 1998).

### Additional Loci Required for Macrophage Survival

Several other *Salmonella* loci are implicated in macrophage survival. The *spv* operon located on the *Salmonella* virulence plasmid is required for systemic infection (Guiney et al., 1995) and encodes SpvB, which functions by ADP-ribosylating actin (Lesnick et al., 2001). SlyA is a transcriptional regulator that has been implicated in resistance to oxidative stress, macrophage survival, and virulence in the mouse model (Buchmeier et al., 1997; Watson et al., 1999). Recent studies (Spory et al., 2002; Stapleton et al., 2002) have identified a number of SlyA-regulated genes in *E. coli* and serovar Typhimurium, including a variety of stress response loci. Thus, the SlyA regulon apparently overlaps with the regulons of both the heat shock  $\sigma$  factor, RpoH, and the general stress response  $\sigma$  factor, RpoS. Null mutations in the *sspJ* gene confer sensitivity

to the redox cycling agent menadione and a virulence defect in macrophages and the animal model of infection (Van der et al., 2001; Van Diepen et al., 2002). The biochemical function of SspJ is yet to be determined. The periplasmic superoxide dismutase SodCI, encoded on the lysogenic bacteriophage Gifsy-2, is required for resistance to phagocytic superoxide (Ho et al., 2002; Uzzau et al., 2002; Krishnakumar et al., 2004). Certain auxotrophic mutations also attenuate macrophage survival and virulence in the animal model (Fields et al., 1986), suggesting that SCVs are limiting for some nutrients.

### The Carrier State and Gallbladder Survival

Approximately 1–4% of individuals infected with *S. typhi* and 0.5% of individuals infected with serovar Typhimurium develop a chronic carrier state infection (Miller and Pegues, 2000). In these chronic infections, the bacteria reside in the gallbladder and development of this state is often associated with abnormalities such as gallstones (Prouty et al., 2002a). Hypothetically, the ability to both resist high concentrations of bile and form biofilms on surfaces such as gallstones is important for establishing the carrier state (Van Velkinburgh and Gunn, 1999; Prouty et al., 2002a; Prouty et al., 2002b). The PhoPQ regulatory system is required for resistance to high concentrations of bile, although none of the known PhoP-regulated loci are individually necessary (Van Velkinburgh and Gunn, 1999). Indeed, the presence of bile decreases transcription of the PhoP-activated gene *pagC* in a PhoP-independent fashion (Prouty et al., 2004b). Other members of the PhoP regulon are apparently unaffected at the transcriptional level. The *yciGFE-katN* operon, which encodes an acid shock protein (YciE) and a non-heme catalase (KatN), is induced in the presence of bile. Also, evidence shows that both the flagellar regulon and the SPI1 TTSS are repressed when bile was added to the medium. Decreased expression of SPI1 is consistent with a previous report that invasion of epithelial cells is decreased in the presence of bile (Prouty and Gunn, 2000). The *marRAB* operon is also induced, the products of which transcriptionally regulate a variety of genes leading to a multiple antibiotic-resistance phenotype, including the *acrAB* operon, which encodes an efflux pump (Prouty et al., 2004a) that likely expels bile from the cytoplasm. AcrAB (Prouty et al., 2004a) and TolQRA, involved in outer membrane integrity and colicin and phage translocation (Prouty et al., 2002b), are required for bile-resistance.

Numerous bacterial species are capable of forming biofilms, or communities of microorganisms, which are often more resistant to

antimicrobial agents, desiccation and harsh environments (Costerton et al., 1995; Davey and O'Toole, 2000). Serovars Typhimurium and Typhi are capable of forming biofilms on human gallstones only when they are grown in the presence of bile (Prouty et al., 2002a). Biofilm formation on gallstones requires LPS, flagella and LuxS, a potential quorum sensing regulator (Prouty et al., 2002a). Interestingly, fimbriae appear to play a negative role in biofilm formation, as a strain containing mutations in *lpfC*, *fim*, *pef* and *agf* actually form biofilms at a faster rate (Prouty et al., 2002a). Also surprisingly, *phoP* mutations enhanced biofilm formation, while the SPI1 TTSS and flagella were required (Prouty and Gunn, 2003). Taken together, these results indicate a complex interaction between bile resistance, gene induction, and biofilm formation.

## Flagella

Most *Salmonella* species, with the exception of serovars Gallinarum and Pullarum, are motile. *Salmonella* motility is mediated by peritrichous flagella, which appear as long thin filaments usually numbering between 5–10 per cell (Macnab, 1996). The flagella structure is composed of nearly 40 gene products and can be separated into three parts, the basal body, a hook, and a long helical filament (Macnab, 1996; Macnab, 2003). The flagellar filament is constructed from ca. 20,000 subunits of a single protein, either FliC or FljB (see Phase Variation).

Flagellar regulation is complex and controlled on multiple levels. The greater than 40 genes involved in flagellar biosynthesis can be separated into three classes on the basis of their regulation. The *flhDC* genes make up the class 1 flagellar genes and encode for positive transcriptional regulators of class 2 flagellar genes, which include the *fliAZY*, *fliEFGHIJK* *fliLMNOPQR*, *flhBAE* and *flgABCDEFGHIIJ* operons (Liu and Matsumura, 1994; X. Liu et al., 1995; Macnab, 1996). Still unclear is whether FlhDC can sense any environmental signal or whether activation of the class 2 genes is simply dependent on the posttranscriptional level of FliDC expression. Numerous global regulatory systems feed into flagellar regulation at the level of FlhDC production (Shin and Park, 1995; Macnab, 1996; Ko and Park, 2000; Adams et al., 2001; Goodier and Ahmer, 2001; Wei et al., 2001; Clegg and Hughes, 2002; Lehnen et al., 2002; Ellermeier and Schlauch, 2003; Tomoyasu et al., 2003).

The class 2 gene product, FliA, is an alternative  $\sigma$  factor that is required for transcription of the class 3 flagellar genes, which include *flgMN*, *motABcheAW*, *tarcheRBYZ*, and *fliCDST* (Macnab, 1996). While FliA is required for maximal

expression of class 3 genes, it is not the sole regulator of class 3 gene expression. FlgM, a class 3 gene product, also regulates expression of class 3 genes by sequestering FliA and blocking activation of the class 3 genes (Gillen and Hughes, 1991; Chilcott and Hughes, 2000). Once the flagellar basal body has been assembled, FlgM is secreted by the assembled flagella, freeing FliA to activate expression of class 3 genes. This mechanism of regulation allows the bacteria to couple the regulation of the class 3 genes to the assembly of the flagella system (Chilcott and Hughes, 2000).

Recently the class 2 flagellar gene *fliZ* has been implicated in expression of the flagellar genes (Ikebe et al., 1999; Kutsukake et al., 1999). Mutants of *fliZ* decrease expression of the class 2 flagellar genes (Ikebe et al., 1999; Kutsukake et al., 1999). FliZ has also been reported to be a positive regulator of *hilA* expression (Eichelberg and Galan, 2000; Lucas et al., 2000; Iyoda et al., 2001). Still unclear is how FliZ increases expression of either the class 2 flagellar genes or *hilA*.

PHASE VARIATION OF THE FLAGELLIN SUBUNIT *Salmonella enterica* subsp. I, II, IIIa and VI are biphasic, capable of producing two antigenically flagellin subunits or H antigens. In serovar Typhimurium, these are encoded by the genes *fliC* and *fljB*, only one of which is expressed at any given moment (Macnab, 1996). The regulation of these two flagellins is controlled by a site-specific recombinase or DNA invertase Hin, which controls expression of *fljAB* by inverting the *fljAB* promoter (Macnab, 1996). When *fljAB* is in the off position, the *fljB* promoter is in the wrong orientation with respect to the *fljAB* genes, and without FljA, *fliC* is expressed, resulting in a flagellar filament constructed of the FliC subunit. When Hin inverts the *fljB* promoter, the *fljAB* genes are expressed and FljA represses transcription of *fliC* resulting in a flagellar filament constructed of the FljB subunit. The FliC and FljB proteins are functionally analogous to each other but are antigenically distinct (Macnab, 1996).

Although flagella are not essential for virulence of serovar Typhimurium in BALB/c mice (Lockman and Curtiss, 1990), they affect interaction with the host at multiple levels (Lockman and Curtiss, 1992; Gewirtz et al., 2001b; Ikeda et al., 2001; Schmitt et al., 2001). The FliC/FljB flagellin proteins interact with TLR5 on the basolateral surface of epithelial cells to activate the NF- $\kappa$ B pathway in epithelial cells (Gewirtz et al., 2001a), leading to secretion of IL-8 and production of human  $\beta$ -defensin 2 (Gewirtz et al., 2000; Takahashi et al., 2001; see Gastroenteritis). FliC has also been shown to be a major CD4<sup>+</sup>

T-cell epitope in mice (McSorley et al., 2000). However, strains that produce only FljB are attenuated during systemic infection in a mouse model (Ikeda et al., 2001).

## Epidemiology

**NONTYPHOIDAL SALMONELLA** Non-Typhi *Salmonella* are estimated to cause 1.4 million cases of gastroenteritis and enteric fever per year in the United States (Mead et al., 1999; Voetsch et al., 2004). This represents 10% of the total food-borne illness in this country and corresponds to an incidence rate of 12.3 cases per 100,000 population (Voetsch et al., 2004). More importantly, of the foodborne diseases, salmonellosis accounts for 26% of hospitalizations (15,000 per year) and 31% of deaths (400–600 per year) (Mead et al., 1999; Voetsch et al., 2004). The incidence of *Salmonella* food-borne disease is seasonal, with most infections occurring in the summer (Anonymous, 1997; D'Souza et al., 2004). The incidence of *Salmonella* infections apparently increased over the last several decades owing largely to the expanding size and centralization of modern agriculture. However, the number of infections per year has remained essentially constant since 1996 (Voetsch et al., 2004). Of course, non-Typhi *Salmonella* are a major cause of morbidity and mortality throughout the world, particularly in children under the age of 5 (Graham, 2002).

*Salmonella* infection is a reportable disease in the United States, but the numbers above are estimates. The CDC receives and characterizes 30,000–40,000 isolates per year. Of the ca. 32,300 isolates received in 2002, serovars Typhimurium, Enteritidis and Newport were the most prevalent, representing 22%, 16% and 13%, respectively (Centers for Disease Control and Prevention, 2002). The remaining serovars that individually represent >2% of isolates were Heidelberg (6.1%), Javiana (3.7%) and Montevideo (2.2%; Centers for Disease Control and Prevention, 2002). Twenty-five percent of the isolates were from patients under 5 years of age. Thirty-seven percent of isolates were evenly distributed among patients 10–49 years of age. The number of isolates from patients >50 years old steadily decreased with the age of the patient (Centers for Disease Control and Prevention, 2002). However, mortality and the proportion of patients who are hospitalized increase significantly in patients over 60 years of age (Kennedy et al., 2004).

A majority of cases are sporadic. However, outbreaks occur frequently. The two largest outbreaks of *Salmonella* in the United States occurred in 1) 1985 when approximately 175,000 people were infected with serovar Typhimurium

from contaminated pasteurized milk (Ryan et al., 1987), and 2) 1994 when approximately 224,000 people were infected with serovar Enteritidis from contaminated ice cream products (Hennessy et al., 1996). *Salmonella* has also been used as a bioterrorism agent. A total of 751 persons were infected with serovar Typhimurium in 10 restaurants in Oregon during October and September 1984 after members of a religious commune had intentionally contaminated salad bars (Torok et al., 1997).

*Salmonella* primarily live in the intestinal tracts of animals (see Habitat). Thus, most infections result from eating contaminated foods from animal origins including meat, poultry, eggs, and dairy products (Miller and Pegues, 2000). Other implicated sources include other animals and fruits and vegetables contaminated with animal feces (Sanchez et al., 2002; Voetsch et al., 2004). Contact with reptiles and amphibians accounts for 6% of human infections (Centers for Disease Control and Prevention, 2003c), including infection with non-subsp. I strains (see Taxonomy). Table 6 lists the most frequent *Salmonella enterica* serovars isolated from symptomatic or non-symptomatic major food animals submitted to the CDC in 2002 (Centers for Disease Control and Prevention, 2003d). Several trends are apparent. Clearly, the most frequent human isolates correlate with the serovars that are commonly found in all three of the major food animals (e.g., serovar Typhimurium). This is also evident in the increase in the frequency of serovar Newport isolates in both cattle (29-fold since 1997;

Table 6. Most frequent clinical and nonclinical isolates from major food animals.

Source	Serovar <sup>a</sup>	% Total isolates for each animal species	
		Clinical <sup>b</sup>	Nonclinical
Bovine	Agona	5	2
	Dublin	5	2
	Kentucky	3	10
	Meleagridis	1	11
	Montevideo	4	26
	Newport	29	2
Chicken	Typhimurium <sup>c</sup>	22	11
	Enteritidis	8	3
	Heidelberg	31	50
	Kentucky	18	14
Porcine	Typhimurium <sup>c</sup>	8	7
	Agona	4	9
	Choleraesuis	14	0
	Derby	17	53
	Typhimurium <sup>c</sup>	28	22

<sup>a</sup>The 3–5 most frequently isolated serovars for either clinical or nonclinical cases are shown.

<sup>b</sup>Clinical indicates that the animal was symptomatic.

<sup>c</sup>Includes variation Copenhagen.

From Centers for Disease Control and Prevention (2003d).

Centers for Disease Control and Prevention, 1998; Centers for Disease Control and Prevention, 2003d) and humans (2.8-fold since 1997; Centers for Disease Control and Prevention, 2003d). However, this correlation is not strict, indicating definite genetic differences among strains, some being more virulent for humans. Indeed, some serovars are “host adapted” (see Habitat). For example, serovar Choleraesuis is a significant pathogen of pigs, but is rarely isolated from human patients (21 of 32,308 isolates in 2002; Centers for Disease Control and Prevention, 2003d).

Consistent with the role of meat products as a source of *Salmonella* infection, the pathogen is routinely recovered from meat samples from United States retail outlets. In several studies, *Salmonella* was recovered from 10–50% of chicken samples (Bokanyi et al., 1990; Izat et al., 1991; Simmons et al., 2003; Rigney et al., 2004), 24–30% of turkey samples (White et al., 2001; Rigney et al., 2004), 10–16% pork products (Duffy et al., 2001; White et al., 2001), and 4–19% of beef samples (White et al., 2001; Fraticchio, 2003; Rigney et al., 2004).

Hard shell eggs are an increasingly important source of *Salmonella* infection. This is due to the emergence of serovar Enteritidis beginning in the 1970s (Guard-Petter, 2001; Centers for Disease Control and Prevention, 2003b). This serovar has been the second leading cause of human infection in the United States and indeed surpassed serovar Typhimurium as the leading cause during the mid-1990s (Centers for Disease Control and Prevention, 2003d). Serovar Enteritidis is unique in that it can infect the ova of hens and can be deposited in eggs before the shell is formed (Guard-Petter, 2001). Inadequate refrigeration during shipment and storage of eggs, and incomplete cooking, allow infection.

The infective dose for foodborne *Salmonella* is difficult to determine and is likely to vary with strain, source (e.g., the particular food matrix could differentially protect from stomach acid), and the underlying health of the individual (Coleman and Marks, 2000; Coleman et al., 2004). Data on infective dose come from both volunteer studies and outbreaks. An analysis of various studies prior to 1982 suggested that the infective dose was  $<10^3$  organisms (Blaser and Newman, 1982). More recent data from outbreaks suggest that the infective dose is surprisingly low. Contaminated paprika led to an outbreak involving several serovars in Germany in 1993. The infective dose was estimated to be 4–45 organisms giving an attack rate of 1 in 10,000 exposed individuals (Lehmacher et al., 1995). Analysis of ice cream samples from the same lots that led to the 1994 outbreak of serovar Enteritidis in the Midwest (Hennessy et al.,

1996), particularly single serving pre-packaged ice cream cones, suggested that the infective dose was 28 cells (Vought and Tatini, 1998). Both of these studies benefited from the fact that the nature of the food, the dried paprika and the frozen ice cream, preserved the viable organisms probably minimizing the change in number from the time of infection to analysis.

The *Salmonella*, like many bacterial pathogens, are becoming increasingly resistant to antibiotics (Parry, 2003). Characterization of isolates received by the CDC in 2001 (Centers for Disease Control and Prevention, 2003a) indicated that 51% of the serovar Typhimurium strains were resistant to at least one of the tested antibiotics and 35% were resistant to at least five antibiotics, most commonly ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline. This multidrug resistant phenotype is indicative of serovar Typhimurium strain DT104, for example, which has recently disseminated worldwide (Davis et al., 2002). Among serovar Enteritidis isolates, 14% were resistant to at least one antibiotic and 1% were resistant to at least five antibiotics (Centers for Disease Control and Prevention, 2003a). For serovar Newport, 35% were resistant to at least one antibiotic while 25% were resistant to at least eight antibiotics. In 1996, none of these multidrug-resistant isolates of serovar Newport were detected (Centers for Disease Control and Prevention, 2003a).

**SEROVARS TYPHI AND PARATYPHI** Serovars Typhi and Paratyphi are human specific pathogens. Thus, infection results from ingestion of water or food contaminated with human feces or close contact with an individual who has typhoid fever or is a chronic carrier (Miller and Pegues, 2000). Because of good public water and sewer systems, typhoid fever is rare in the United States, with an estimated 825 cases per year (Mead et al., 1999). Greater than 70% of these cases are in people who acquired the infection upon visiting an endemic area (Mead et al., 1999). However, outbreaks do occur in the United States, for example, from imported contaminated fruit (Katz et al., 2002), or from food service workers who are chronic carriers (Lin et al., 1988; Birkhead et al., 1993).

Worldwide, typhoid fever is a major health problem with an estimated 17 million cases per year resulting in 600,000 deaths (Pang et al., 1998). The infection is particularly prevalent in Asia and Africa (Pang et al., 1998) with rates as high as 980 per 100,000 population in Delhi, India, for example (Parry et al., 2002). Infection is reportedly highest in 3–19 year-olds (Pang et al., 1998), but more recent data suggest that 44–54% of infections occur in children under 5 years

of age (Graham, 2002). Reliable data is difficult to attain because of limited health care, public health infrastructure, and microbiological work-up in many of these developing nations.

The case fatality rate for untreated typhoid fever is 10–20%, but the advent of antibiotic treatment has reduced the fatality rate to less than 1% (Miller and Pegues, 2000; Maskalyk, 2003). Chloramphenicol has historically been the drug of choice, but resistance to chloramphenicol, ampicillin, and trimethoprim/sulfamethoxazole has been rising since the 1980s with up to 65% of strains isolated in India being multidrug resistant, for example (Pang et al., 1998). Fluoroquinolones are very effective (Parry et al., 2002), but resistance to these drugs is also being seen (Threlfall, 2002).

### Disease

**NONTYPHOIDAL *SALMONELLA*** Infection by non-typhoid *Salmonella* usually results in self-limiting acute gastroenteritis (Shere et al., 1998; Miller and Pegues, 2000). After an incubation period of 6–48 h, there is often an onset of nausea and vomiting followed by abdominal pain and loose, watery diarrhea. The extent of diarrhea is variable, and blood and mucous in the stool are not uncommon. The abdominal pain can range from colicky discomfort to pseudoappendicitis. Approximately 50% of patients will have a slight fever (<39°C) and chills but body temperature will return to normal in 1–2 days. The diarrhea lasts 3–7 days, although organisms can be shed for 4–5 weeks; longer in neonates. Approximately 6% of patients will develop Reiter's syndrome or reactive arthritis (Barth and Segal, 1999), most likely if the individual expresses HLA-B27 (Yu and Kuipers, 2003). Diagnosis is based on history and stool culture (see Isolation). Patients with simple gastroenteritis do not require treatment other than oral rehydration therapy. Indeed, the vast majority of these patients will not seek medical treatment and will never confirm that they were infected with *Salmonella*; hence only a small fraction of *Salmonella* cases are actually reported.

In addition to simple gastroenteritis, infection with non-Typhi *Salmonella* can be more severe, resulting in bacteremia, fever, or other complications (Shere et al., 1998; Miller and Pegues, 2000; Vugia et al., 2004). Certain individuals are particularly susceptible to more severe *Salmonella* infection, including those <1 year-old, the elderly, and immunocompromised patients, especially HIV infected patients, who often suffer from recurrent *Salmonella* bacteremia. One to four percent of immunocompetent individuals and an increased percent of those with weakened immune systems have sustained bacteremia and

fever. Once the infection has become systemic, the bacteria can colonize, albeit rarely, essentially any tissue in the body (Shere et al., 1998), including bone, meninges, lungs, and brain. *Salmonella* is a minor, but not insignificant cause of endocarditis, particularly in cases involving structurally abnormal heart valves. The bacteria are also responsible for a significant fraction of infective aortic aneurysms, indicative of the predilection of *Salmonella* to infect vascular tissue. These vascular infections have a high mortality rate (Fernandez Guerrero et al., 2004). The patients with significant bacteremia and extraintestinal infections are the most likely to seek medical care, and antibiotic treatment is warranted.

**SEROVARS TYPHI AND PARATYPHI** Serovars Typhi and Paratyphi are host adapted to humans, causing classic typhoid (or paratyphoid) fever (Shere et al., 1998; Miller and Pegues, 2000). After an incubation period of 5–21 days, some patients will have diarrhea and pain that may last several days. Individuals with or without diarrhea then develop fever that rises over 2–3 days to 39–40°C. Other symptoms can include chills, myalgias, abdominal pain, headache, cough, sore throat, and “rose spots,” a maculopapular rash seen on the trunk of 30% of patients. Psychosis and confusion occur in 5–10% of patients. Constipation is not uncommon. Perforation of the terminal ileum or proximal colon occurs in 3–10% of patients and is a significant cause of mortality. Symptoms are more severe in Typhoid fever versus Paratyphoid fever and are more severe in infants. For those untreated patients that survive, symptoms last approximately 4 weeks, although weakness may be evident for months. Ten percent of individuals will suffer a relapse. One to four percent of patients will develop long-term carriage (see Carrier State). Diagnosis is based on isolation of the bacteria, usually from blood or stool (see Isolation). Antibiotic treatment (see Epidemiology) significantly reduces the mortality rate and the duration of fever.

Vaccination against typhoid is recommended for travelers to endemic areas and for laboratory personnel who work with serovar Typhi (Miller and Pegues, 2000). There are three commercially available typhoid vaccines: a heat-phenol inactivated parenteral vaccine, an oral live attenuated Ty21a vaccine, and a more recent purified Vi capsular parenteral vaccine. All three have shown variable efficacies from approximately 45–95%. The heat-killed vaccine has the potential for more serious side effects than the other two. The Vi vaccine is gaining support and is being suggested for more widespread use (Pang et al., 1998). A new conjugate Vi vaccine is particularly promising (Lin et al., 2001; Mai et al., 2003).

**DISEASE IN ANIMALS** The *Salmonella* live primarily in the intestines of animals and cause significant disease in these hosts. These include the major agricultural animals and, therefore, *Salmonella* has significant consequences for production. The various serovars can be host adapted or restricted and, as is seen in humans, the nature and degree of disease is dependent on the specific serovar and host involved. Animals can be chronically infected and serve as important sources of organisms, or the infections can be acutely lethal. For example, serovar Dublin causes diarrhea in young calves but can also cause abortion in pregnant cows with no other clinical symptoms (Santos et al., 2001). Serovar Choleraesuis causes enterocolitis or septicemia in swine. Serovar Gallinarium causes fowl typhoid, a persistent infection. Serovar Enteritidis infects the oviduct of chickens, often with no clinical symptoms, yet the eggs become infected prior to the deposition of the shell (Guard-Petter, 2001). This is an important source of infection for humans.

## Applications

Detailed knowledge of *Salmonella* physiology, genetics, pathogenesis, and host immune response has made various attenuated serovar Typhimurium or serovar Typhi strains favorite candidates for live attenuated vaccines for delivery of exogenous antigens (e.g., Garmory et al., 2002; Garmory et al., 2003). The goal is to stimulate the mucosal immune response, the first line of defense against most pathogens.

A second major application of *Salmonella* is the Ames Test (Gee et al., 1994). Bruce Ames developed the concept that carcinogens can be assayed by measuring mutagenic activity in bacteria, rather than by directly testing carcinogenesis in animals. Compounds, usually after treatment with liver extract to mimic metabolism in the human body, are mixed with one of several serovar Typhimurium strains that are histidine-requiring auxotrophs. The number of prototrophic revertants is a measure of mutagenic potential of the compound.

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## The Genus *Klebsiella*

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### Taxonomic History and Structure

#### Historical Developments

The genus *Klebsiella*, in the family Enterobacteriaceae, was named by Trevisan (1885) to honor the German microbiologist Edwin Klebs (1834–1913). The type species is *Klebsiella pneumoniae* (Schroeter, 1886; Trevisan, 1887). The first *Klebsiella* species ever described was a capsulated bacillus from patients with rhinoscleroma (Von Frisch, 1882). The organism was named “*Klebsiella rhinoscleromatis*” by Trevisan (1887).

Abel (1893) observed a capsulated bacillus, “*Bacillus mucosus ozaenae*” from the nasal secretion of patients with ozaena. The bacterium was later transferred to the genus *Klebsiella* as *K. ozaenae* (Bergey et al., 1925).

Friedländer (1882) described a bacterium from the lungs of a patient who had died of pneumonia. The organism was named “*Hyalococcus pneumoniae*” (Schroeter, 1889) and *Klebsiella pneumoniae* (Trevisan, 1887). Considerable confusion occurred for many years since the organism could not be objectively separated from the bacterium Escherich described as “*Bacterium lactis aerogenes*” (Escherich, 1885) and which was renamed “*Bacillus aerogenes*” (Kruse, 1896), “*Aerobacter aerogenes*” (Kruse, 1896), and later *Enterobacter aerogenes* (Hormaeche and Edwards, 1960). Both *K. pneumoniae* and “*Aerobacter aerogenes*” fermented many carbohydrates (often with gas production), gave a positive Voges-Proskauer reaction and reacted with *Klebsiella* capsular antisera. A step forward was taken when Møller (1955) introduced the decarboxylase tests. *Klebsiella pneumoniae* was defined as nonmotile and ornithine decarboxylase negative, whereas “*A. aerogenes*” was defined as motile or nonmotile and ornithine decarboxylase positive (Hormaeche and Edwards, 1958). To reduce confusion in communication, a new genus *Enterobacter* was formed (Hormaeche and Edwards, 1960) in which “*A. aerogenes*” was transferred as *E. aerogenes*. Historically, this separation was probably arbitrary (authentic strains being unavailable), but these

redescriptions and the designation of neotype strains solved the problem for most microbiologists. However, *E. aerogenes* is much closer to *Klebsiella* species than to *Enterobacter cloacae* on the basis of phenotypic traits and DNA relatedness (Bascomb et al., 1971; Brenner et al., 1972; Steigerwalt et al., 1976; Izard et al., 1980) and 16S rRNA sequence (Boye and Hansen, 2003). The transfer of *E. aerogenes* to the genus *Klebsiella* as *K. mobilis* has been proposed (Bascomb et al., 1971). Nomenclatural confusion remained for some time in the United Kingdom where a biogroup of *K. pneumoniae* was named “*Klebsiella aerogenes*” (Taylor et al., 1956). Cowan et al. (1960) subdivided *K. pneumoniae* sensu lato into *K. pneumoniae* (sensu stricto), “*K. aerogenes*,” “*K. edwardsii* subsp. *Edwardsii*,” and “*K. edwardsii* subsp. *Atlantae*.” Authentic or typical strains of *K. pneumoniae*, “*K. edwardsii*” and “*K. aerogenes*” cannot be differentiated by DNA relatedness or protein electrophoresis and thus belong to a single species, *K. pneumoniae* (Brenner et al., 1972; Jain et al., 1974; Ferragut et al., 1989). Although their biochemical patterns allow easy identification, *K. ozaenae* and *K. rhinoscleromatis* cannot be separated from *K. pneumoniae* by DNA relatedness (Brenner et al., 1972). For this reason, *K. ozaenae* and *K. rhinoscleromatis* were treated as subspecies of *K. pneumoniae* in the *Bergey's Manual of Systematic Bacteriology* (Ørskov, 1974).

Flügge (1886) described “*Bacillus oxytocus perniciosus*” from old milk. This organism was named “*Aerobacter oxytocum*” (Bergey, 1923) and *Klebsiella oxytoca* (Lautrop, 1956). For many years, the existence of this indole positive species was questioned, and it was often considered a biogroup of *K. pneumoniae* (Edwards and Ewing, 1972; Ørskov, 1974). DNA relatedness studies showed *K. oxytoca* to be clearly distinct from *K. pneumoniae* (Jain et al., 1974; Brenner et al., 1977).

The use of carbon source utilization tests and DNA hybridization allowed the delineation and characterization of two new species primarily isolated from environmental samples: *K. terrigena* (Izard et al., 1981) and *K. planticola*

(Bagley et al., 1981). The species described as *K. trevisanii* (Ferragut et al., 1983) was found to be identical to *K. planticola* (Gavini et al., 1986). The name “*K. ornithinolytica*” was proposed for ornithine-positive, indole-positive strains of *Klebsiella* (Sakazaki et al., 1989). The distinctness of this bacterial group from *K. planticola* needs to be confirmed since different results have been obtained in another laboratory (Farmer et al., 1985).

The move of *K. planticola*, *K. ornithinolytica* and *K. terrigena* to a new genus, *Raoultella* was recently proposed (Drancourt et al., 2001). However, the validity of this proposal is dubious since phylogenetic analysis of 16S rRNA gene sequence did not provide strong support for the monophyly of *Raoultella* (Drancourt et al., 2001; Boye and Hansen, 2003) and since *rpoB* sequence data failed to group *Raoultella* species into a single cluster (Drancourt et al., 2001).

The agent of donovanosis, *Calymmatobacterium granulomatis*, was found to be very closely related to *Klebsiella pneumoniae* on the basis of the 16S rRNA gene sequence (Carter et al., 1999; Kharsany et al., 1999) and on the phosphate porin (*phoE*) gene sequence (Bastian and Bowden, 1996; Carter et al., 1999). *Calymmatobacterium granulomatis* was proposed to be transferred to the genus *Klebsiella* as *Klebsiella granulomatis* (Carter et al., 1999), a proposal that had also been made by Richens (1985) given the similarity of pathological aspects of *C. granulomatis*, *K. pneumoniae* subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *ozaenae*. However, whether *Klebsiella granulomatis* is a genomic species distinct from *K. pneumoniae* is unknown. Instead, partial *rpoB* sequencing (Drancourt et al., 2001) confirmed *phoE* findings that *K. granulomatis* is very close to *K. pneumoniae*. Thus, *K. granulomatis* may correspond to a subspecies of *K. pneumoniae*.

The genetic diversity of *K. pneumoniae* and *K. oxytoca* species has been investigated using *gyrA* and *parC* gene nucleotide diversity, random amplified polymorphic DNA (RAPD), and ribotyping (Brisse and Verhoef, 2001). Three sequence clusters, named “KpI,” “KpII” and “KpIII,” were evidenced within *K. pneumoniae*, which did not correspond to the known subspecies, and two sequence clusters, KoI and KoII, were found within *K. oxytoca*. The genetic distinctness of these five clusters was confirmed using amplified fragment-length polymorphism (AFLP; Jonas et al., 2004). Two *K. oxytoca* groups were also found on the basis of partial sequences of the *rpoB* and 16S rRNA genes and the chromosomal  $\beta$ -lactamase genes *bla*<sub>OXY-1</sub> and *bla*<sub>OXY-2</sub> (Granier et al., 2003b). These groups correspond to KoI and KoII, respectively (S. Brisse, unpublished observation). In addition, there is

evidence that more phylogenetic groups exist within *K. pneumoniae* (Jonas et al., 2004) as well as within *K. oxytoca* (Granier et al., 2003a). Utilization of D-adonitol as sole carbon source was found to differ between KpI (all strains being positive) and KpIII (all strains being negative), while KpII was heterogeneous in this respect. DNA-DNA reassociation studies will be needed to determine whether these *K. pneumoniae* and *K. oxytoca* phylogenetic groups represent distinct genomic species.

## Taxonomic Structure

The taxonomic structure of the genus *Klebsiella* that is currently widely accepted is given below. Uncertain points that still need to be clarified are the distinction between *K. planticola* and *K. ornithinolytica* and the phylogenetic distinctness of *K. granulomatis* and *K. pneumoniae*. The inclusion of *E. aerogenes* as *K. mobilis* is not consensual. Note that the definitions of *K. pneumoniae* subspecies are based on pathogenesis criteria and not on genomic distinctness.

1. *Klebsiella pneumoniae* (Schroeter, 1886; Trevisan, 1887). The type strain is ATCC 13883 (=NCTC 9633, CDC 298-53, CIP 82.91). This species contains three subspecies:

1a. *Klebsiella pneumoniae* subspecies *pneumoniae* (Schroeter, 1886; Ørskov, 1984a). The type strain is the same as for the species.

1b. *Klebsiella pneumoniae* subspecies *ozaenae* (Abel, 1893; Ørskov, 1984a), “*Bacillus mucosus ozaenae*” (Abel, 1893). The type strain is ATCC 11296 (= NCTC 5050, CIP 52.211).

1c. *Klebsiella pneumoniae* subspecies *rhinoscleromatis* (Trevisan, 1887; Ørskov, 1984a); *Klebsiella rhinoscleromatis* (Trevisan, 1887). The type strain is ATCC 13884 (= NCTC 5046, CIP 52.210).

2. *Klebsiella granulomatis* (Aragão and Vianna, 1913; Carter et al., 1999). Basonym: *Calymmatobacterium granulomatis* Aragão and Vianna, 1913; Approved Lists, 1980). Other synonyms: “*Encapsulatus inguinalis*” (Bergey, 1923), “*Klebsiella granulomatis*” (Bergey et al., 1925), “*Donovania granulomatis*” (Anderson et al., 1944). No type culture is currently available.

3. *Klebsiella oxytoca* (Flügge, 1886; Lautrop, 1956); “*Bacillus oxytocus perniciosus*” (Flügge, 1886). The type strain is ATCC 13182.

4. *Klebsiella planticola* (Bagley et al., 1981). The type strain is ATCC 33531 (= V-236, CDC 4245-72, CIP 100751). *Klebsiella trevisanii* (Ferragut et al., 1983; Phenon K of Gavini et al., 1977) is a junior subjective synonym of *K. planticola* (Gavini et al., 1986).

5. *Klebsiella ornithinolytica* (Sakazaki et al., 1989). The type strain is ATCC 31898 (NIH 90-72). The CDC group called “*Klebsiella* Group 47” corresponds to this species (Sakazaki et al., 1989).

6. *Klebsiella terrigena* (Izard et al., 1981), phenon L (Gavini et al., 1977). The type strain is ATCC 33257 (= CIP 80-07, CUETM 77-176).

7. *Klebsiella mobilis* (Bascomb et al., 1971), *Enterobacter aerogenes* (Kruse, 1896; Hormaeche and Edwards, 1960). The type strain is ATCC 13048 (= NCTC 10006, CDC 819-56, CIP

60.86). *Klebsiella mobilis* (*Enterobacter aerogenes*) will not be treated below (except for purpose of differentiation).

8. *Klebsiella variicola* Rosenblueth et al., 2004. The type strain is F2R9 (= ATCC BAA-830 = CFNE 2004). Corresponds to phylogenetic group KpIII of Brisse and Verhoef (2001).

## Isolation

### Underlying Principles

*Klebsiella* spp. grow readily on ordinary media commonly used to isolate Enterobacteriaceae, e.g., nutrient agar, tryptic casein soy agar, bromocresol purple lactose agar, blood agar, as well as more differential plating media for Enterobacteriaceae, such as Drigalski agar, MacConkey agar, eosin-methylene blue agar (EMB), and bromo-thymol blue agar (BTB). *Klebsiella pneumoniae* and *K. oxytoca* colonies are lactose positive, more or less dome-shaped, 3–4 mm in diameter after overnight incubation at 30°C or 37°C, with a mucoid aspect and sometimes stickiness, depending on the strain and the composition of the medium. *Klebsiella planticola* and *K. terrigena* colonies are also lactose positive, 1.5–2.5 mm in diameter, dome-shaped, with a weakly mucoid aspect. *Enterobacter aerogenes* (*K. mobilis*) colonies often have the same morphology. *Klebsiella ozaenae*, *K. rhinoscleromatis* and occasionally *K. pneumoniae* K1 grow more slowly on the same media, yielding voluminous, rounded, very mucoid, translucent and confluent colonies in 48 h at 30°C or 37°C (Ørskov, 1981; Richard, 1982). Similar colonies indistinguishable from those of *Klebsiella* may be formed by other genera of the Enterobacteriaceae, particularly *E. coli* mucoid varieties with capsular K antigens (Kauffmann, 1949; Ørskov and Fife-Asbury, 1977).

Almost all *Klebsiella* strains grow in minimal medium with ammonium ions or nitrate as sole nitrogen source and a carbon source (see below) without growth factor requirement. Some *K. pneumoniae* K1 isolates require arginine or adenine or both as growth factors. *Klebsiella pneumoniae* subsp. *rhinoscleromatis* requires arginine and uracil. Growth factor requirements of *K. pneumoniae* subsp. *ozaenae* are not fully determined (leucine, cysteine and methionine are stimulatory; O. Bouvet and P. A. D. Grimont, unpublished observations). In these requirements, ornithine can replace arginine.

*Klebsiella* strains can be conserved at room temperature in meat extract semisolid agar, or at –80°C in a broth medium with 10–50% (v/v) glycerol, or freeze-dried.

*Klebsiella granulomatis* has not been grown axenically in artificial media. In vivo cultures in the yolk sac or brain of chick embryo have been

achieved. In vitro cultures use fresh mononuclear cells (Kharsany et al., 1997) or the Hep-2 human epithelial cell line (Carter et al., 1997).

### Selective Media

Several selective differential media for the isolation and enumeration of *Klebsiella* spp. were proposed in clinical, industrial and natural environments. The ability to utilize citrate (Cooke et al., 1979) or *myo*-inositol (Legakis et al., 1976) has been applied to selective medium formulation. Resistance of *Klebsiella* spp. to methyl violet (Campbell and Roth, 1975), double violet (Campbell et al., 1976), potassium tellurite (Tomas, 1986), and carbenicillin (Thom, 1970) has been used in selective media.

Thom (1970) developed a medium based on the MacConkey agar in which lactose is replaced by inositol, with the addition of 100 µg of carbenicillin per ml. Bagley and Seidler (1978a) devised a similar medium with only 50 µg of carbenicillin per ml.

MacConkey-inositol-carbenicillin Agar (Bagley and Seidler, 1978a)

MacConkey agar base (Difco)	40 g
<i>myo</i> -Inositol	10 g
Distilled water to	1 liter

Autoclave at 121°C for 15 min, cool to 50°C in a water bath, add 0.05 g of carbenicillin dissolved in 5 ml of sterile distilled water, and mix and dispense into sterile Petri dishes. Keep at 4°C and use within three days.

About 95% of pink-to-red colonies were verified to be *Klebsiella* spp., whereas only 1% of yellow background colonies were *Klebsiella*.

Since about 10% of *Klebsiella* strains are susceptible to 50 µg per ml of carbenicillin, the antibiotic was replaced in the above formula by tellurite (K<sub>2</sub>TeO<sub>3</sub>; Tomas, 1986), which is a strong inhibitor of phosphate transport in *Escherichia coli*. Minimal inhibitory concentrations of tellurite were 100 or 200 µg per ml for *K. pneumoniae* subsp. *pneumoniae*, *K. oxytoca*, *K. planticola* and *K. terrigena*, 10 µg per ml for *K. pneumoniae* subsp. *ozaenae*, and 1–3 µg per ml for other Enterobacteriaceae (Tomas, 1986). MacConkey-inositol-potassium tellurite agar is prepared by suspending 40 g of MacConkey agar base in 1 liter of distilled water. The preparation is autoclaved at 121°C for 15 min, cooled to 50°C, then supplemented with filter sterilized *myo*-inositol (final concentration, 10 mM) and potassium tellurite (final concentration 3 µg/ml). After mixing, the medium is dispensed in Petri dishes. This medium keeps well at 4°C for more than two months (Tomas, 1986). In a field test, 77% of pink-to-red colonies were confirmed as *Klebsiella* spp. However, the efficiency of plating was about 1% (Dutka et al., 1987), and some *Kleb-*

*siella* strains may be too susceptible to tellurite to grow on this medium.

Wong et al. (1985) devised a minimal medium in which the carbon source is lactose and the nitrogen source is potassium nitrate. Inhibitory compounds were deoxycholate, neutral red, and crystal violet.

Minimal Medium with Lactose, Nitrate, Deoxycholate, Neutral Red and Crystal Violet (Wong et al., 1985)

Hot deionized water	800 ml
Lactose	5.0 g
Sodium deoxycholate	1.0 g
Na <sub>2</sub> HPO <sub>4</sub>	0.7 g
KNO <sub>3</sub>	1.08 g
NaH <sub>2</sub> PO <sub>4</sub>	0.3 g
MgSO <sub>4</sub> · H <sub>2</sub> O	0.2 g
MnSO <sub>4</sub>	0.1 g
FeCl <sub>2</sub>	0.005 g
ZnCl <sub>2</sub>	0.005 g
CoCl <sub>2</sub>	0.005 g
Mo(OH) <sub>2</sub>	0.005 g

Dissolve and filter through Whatmann no. 2 filter paper. Adjust pH to 6.8 with 1 N NaOH. Then add the following: neutral red (0.03 g), crystal violet (0.004 g), and Noble agar (15 g). Boil to dissolve the agar, autoclave at 121°C for 15 min, and cool and dispense in Petri dishes. Incubate inoculated plates at 35°C for 36 h.

On this medium, *K. pneumoniae* and *K. oxytoca* grow as convex, and 1- to 2 mm-diameter, rather mucoid pink-to-red colonies, or larger, more watery pale red colonies with a dark red center. Non-*Klebsiella* either fail to grow or give colorless colonies (Wong et al., 1985).

Bruce et al. (1981) devised an agar medium combining Koser citrate and raffinose (carbon sources) and ornithine and low pH (for ornithine decarboxylase).

Acidic Koser Citrate Medium with Ornithine and Raffinose (Bruce et al., 1981)

Agar (BBL)	20.0 g
Koser citrate (Difco)	5.7 g
Phenol red (5% in 50% ethanol)	10 ml
L-Ornithine	10.0 g
Raffinose	7.0 g

Adjust pH to 5.6 with 1 N HCl and autoclave the medium at 121°C for 15 min. Cool and dispense into Petri dishes.

*Klebsiella* strains grow as yellow mucoid colonies. Other members of the Enterobacteriaceae either do not grow or produce small colorless, pink, red or orange colonies.

For the isolation of *K. pneumoniae* and *K. oxytoca* from human feces, Van Kregten et al. (1984) have developed a medium based on the presence of two carbon sources, citrate and inositol, without inhibitor. The medium consists of Simmons citrate agar with 1% inositol. The medium can be stored at 4°C. *Klebsiella* spp. appear as yellow, dome-shaped, often mucoid colonies, whereas *E.*

*coli* appears as tiny, watery colonies. Apart from some *Enterobacter* strains, no other bacteria grow on the medium.

## Identification

The general methods used for the identification of *Klebsiella* species and other Enterobacteriaceae can be found in Ewing (1986) and Farmer et al. (1980). However, other tests are extremely useful in enterobacterial taxonomy. These include carbon source utilization tests, glucose oxidation (in the presence or absence of added pyrroloquinoline quinone), gluconate- and 2-ketogluconate dehydrogenase tests (Bouvet et al., 1989), and tetrathionate reductase and β-xylosidase tests (Grimont and Grimont, 1992a).

Richard (1985) recommended the use of brilliant green-bile-lactose-L-tryptophan broth (Diagnostics Pasteur, Marnes-la-Coquette, France) to detect growth, gas production from lactose, and indole production at 44.5°C.

Urease is best tested on the urea-indole medium (Diagnostics Pasteur, Marnes-la-Coquette, France; Richard, 1975). This orange-colored medium is inoculated with a loopful of culture from a sugar-containing medium (e.g., Worfel-Ferguson, Hajna, or Kligler agar). After 30 min to a few h of incubation in a water bath at 37°C, the medium turns purple if urea has been split into ammonium carbonate (Richard, 1975).

## Conditions of Incubation

Good results are obtained when *Klebsiella* cultures are incubated at 30–35°C. Although *Klebsiella* isolates from clinical specimens often have an optimal temperature for growth near 37°C, some tests (e.g., Voges-Proskauer test) are more frequently positive at lower temperatures.

## Identification of *Klebsiella* at the Genus Level

There is no single test able to separate the present genus *Enterobacter* from the genus *Klebsiella*. *Enterobacter aerogenes* and *E. gergoviae* are metabolically as active as *Klebsiella* species. No strain of *Klebsiella* is motile. However, non-motile strains of *Enterobacter* occur. Adonitol is generally utilized and fermented by *Klebsiella* and not by *Enterobacter* (except *E. aerogenes* and occasionally *E. cloacae*). D-Arabitol is generally utilized by *Klebsiella* and not by *Enterobacter* (except *E. aerogenes*, *E. gergoviae*, and occasionally *E. cloacae* or *E. agglomerans*). D-Sorbitol is utilized and fermented by some *Klebsiella* species or strains and never by *Enter-*

*obacter* species. Urease is produced by *K. pneumoniae*, *K. oxytoca*, *K. planticola* and *K. terrigena* and not produced by all *Enterobacter* species except *E. gergoviae*. The decarboxylase pattern is most useful in identification of the genus *Klebsiella*. All *Klebsiella* species (except *K. mobilis* and *K. pneumoniae* subsp. *rhinoscleromatis* and some strains of subspecies *ozaenae*) produce a lysine decarboxylase (LDC) but no ornithine decarboxylase (ODC; except strains referred to as "*K. ornithinolytica*") or arginine dihydrolase (ADH). *Enterobacter* species either are LDC+, ODC+, and ADH-; or LDC-, ODC+, and ADH+; or LDC-, ODC+, and ADH-; or LDC-, ODC-, and ADH-. Finally, although capsulated *Enterobacter* strains (mostly *E. aerogenes*) occur, the presence of a capsule in a Voges-Proskauer positive, LDC+, ODC-, ADH-, and nonmotile Gram-negative Enterobacteriaceae points the identification toward the genus *Klebsiella*.

The growth of *Klebsiella* on a carbohydrate-rich medium, such as bromothymol blue lactose agar (Ørskov, 1981), Hajna or Kligler medium, or Worfel-Ferguson medium (Edwards and Ewing, 1972) gives a better development of the capsule than a carbohydrate-poor medium.

Worfel-Ferguson Medium (Edwards and Ewing, 1972)

NaCl	2 g
K <sub>2</sub> SO <sub>4</sub>	1 g
MgSO <sub>4</sub>	0.25 g
Sucrose	20 g
Yeast extract (Difco)	2 g
Agar (Difco)	15 g
Distilled water to	1000 ml

Autoclave at 120°C for 15 min. Use this medium with or without agar. After overnight incubation at 37°C, observe the capsules under the microscope with China ink.

*Klebsiella* are straight Gram-negative rods, 0.3–1 µm in diameter and 0.6–6 µm in length, often surrounded with a capsule (Ørskov, 1984a). The large mucoid colonies often contain cells with large capsules and those of *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* are often voluminous. Usually the methyl red test is negative and the Voges-Proskauer (VP) test is positive, meaning that acetoin and 2,3-butanediol are produced from glucose fermentation and that neutral end products predominate over the acidic end products. Some strains of *K. rhinoscleromatis* do not form acetoin and 2,3-butanediol, other strains produce acetoin and 2,3-butanediol in such small amounts that the methyl red reaction remains positive, and in other strains, the acetoin disappears before the VP reaction is tested, so both tests may be positive or both tests negative (Ørskov, 1984a). Richard's modification of the VP test (Grimont and Grimont, 1992a) gives

most often positive results with *Klebsiella* strains (except *K. pneumoniae* subsp. *ozaenae* and subsp. *rhinoscleromatis*). Esculin is hydrolyzed. Almost all strains in the genus *Klebsiella* (including or not including *K. mobilis*) utilize the following compounds as sole carbon and energy sources (provided growth factor requirements of some strains be met): *N*-acetyl-D-glucosamine, L-alanine, L-arabinose, D-arabitol, L-aspartate, D-cellobiose, citrate, D-fructose, L-fucose, fumarate, D-galactose, gentiobiose, D-gluconate, D-glucosamine, D-glucose, DL-glycerate, glycerol, *myo*-inositol, 2-ketogluconate, DL-lactate, lactose, D-malate, L-malate, maltose, maltotriose, D-mannitol, D-mannose, D-melibiose, 1-*O*-methyl-β-glucoside, L-proline, D-raffinose, D-ribose, L-serine, D-trehalose, and D-xylose (P. A. D. Grimont and E. Ageron, unpublished observations). H<sub>2</sub>S, β-glucuronidase, phenylalanine-, and tryptophan deaminase are not produced; DNA and tributyrin are not hydrolyzed; 2,5-diketogluconate is not formed from 2-ketogluconate (Bouvet et al., 1989). Almost no strain of *Klebsiella* (including or not including *K. mobilis*) can utilize the following compounds as sole carbon and energy sources after a 4-day incubation: betaine, caprate, caprylate, glutarate, itaconate, 3-phenylpropionate, and propionate (P. A. D. Grimont and E. Ageron, unpublished observations).

### Identification of *Klebsiella* Species

**IDENTIFICATION BASED ON PHENOTYPIC METHODS.** The phenotypic characteristics that are most useful for the identification of *Klebsiella* species (including *K. mobilis*) are given in Table 1. Results of carbon source utilization tests have been obtained with Biotype-100 strips (BioMérieux, France) together with a minimal medium containing 16 growth factors (Grimont and Grimont, 1992a). The strips were examined for visual growth after two (early reading) and four (late reading) days. These results of carbon source utilization tests are unpublished observations of P. A. D. Grimont and E. Ageron.

Table 1 obviously shows that *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* are biotypes of *K. pneumoniae* subsp. *pneumoniae* with less nutritional versatility. There is no substrate utilized by subspecies *ozaenae* and *rhinoscleromatis* that are not utilized by subspecies *pneumoniae*. For this reason, it is safer to check the identification of these less active subspecies by capsular typing (almost all *K. pneumoniae* subsp. *rhinoscleromatis* strains are of serotype K3, whereas klebsiellae of serotype K4 are found almost exclusively in *K. pneumoniae* subsp. *ozaenae* and represent the vast majority of strains of this subspecies).

Table 1. Phenotypic characteristics of the *Klebsiella* species and *K. pneumoniae* subspecies.

	<i>K. pneumoniae</i> subsp.							
	<i>pneumoniae</i>	<i>ozaenae</i>	<i>rhinoscleromatis</i>	<i>oxytoca</i>	<i>terrigena</i>	<i>planticola</i>	<i>ornithinolytica</i>	<i>mobilis</i> ( <i>E. aerogenes</i> )
Growth at 5°C	-	-	-	-	+	+	+	+
Growth at 41°C	+	+	+	+	-	d	+	+
Growth at 44.5°C	+	ND	ND	d	-	-	ND	ND
Motility	-	-	-	-	-	-	-	+
Urea hydrolyzed	+	d	-	+	+	+	+	-
Pectate hydrolyzed	-	-	-	+	-	-	-	-
ONPG test	+	+	-	+	+	+	+	+
Indole produced	-	-	-	+	-	d	+	-
Voges-Proskauer test	+	-	-	+	+	+	+	+
Malonate test	+	-	+	+	+	+	+	+
Lysine decarboxylated	+	d	-	+	+	+	+	+
Ornithine decarboxylated	-	-	-	-	-	-	+	+
Glucose dehydrogenase without added PQQ	+	-	-	-	-	-	ND	+
with PQQ added	+	-	-	+	+	+	ND	+
Gluconate dehydrogenase	+	-	-	-	+	+	ND	+
Utilization of								
Adonitol	d	+	(+)	+	+	+	+	+
D-Alanine	+	+	-	+	+	+	+	+
L-Arabitol	-	-	-	d	-	-	-	-
Benzoate	d	-	-	d	d	+	+	+
<i>m</i> -Coumarate	d	d	-	+	-	+	+	-
Dulcitol	d	-	-	d	-	d	d	d
<i>i</i> -Erythritol	-	-	-	-	-	(d)	d	-
D-Galacturonate	+	+	-	+	+	+	+	+
Gentisate	-	-	-	+	d	-	-	+
D-Glucuronate	+	+	-	+	+	+	+	+
Histamine	-	-	-	-	d	+	d	(+)
3-Hydroxybenzoate	-	-	-	+	(+)	-	-	+
4-Hydroxybenzoate	+	-	-	+	+	+	+	d
5-Ketogluconate	d	-	-	+	+	+	+	d
Lactulose	+	d	-	+	(+)	+	+	(+)
Malonate	d	-	d	(d)	-	(d)	d	d
Maltitol	+	+	-	+	+	+	+	+
D-Melezitose	-	-	-	d	+	-	-	-
1- <i>O</i> -Methyl-β-galactoside	+	+	-	+	+	+	+	+
3- <i>O</i> -Methyl-D-glucose	-	-	-	-	+	+	+	-
1- <i>O</i> -Methyl-α-D-glucoside	d	+	-	+	+	+	+	+
Mucate	+	d	-	+	+	+	(+)	+
Palatinose	+	+	-	+	+	+	+	+
Phenylacetate	d	d	-	+	+	+	+	+
Protocatechuate	+	d	-	+	+	+	+	+
Putrescine	d	d	-	(+)	(+)	+	+	d
Quinate	+	d	-	+	+	+	+	+
L-Rhamnose	+	d	+	+	+	+	+	+
D-Saccharate	+	d	+	+	+	+	(+)	+
D-Sorbitol	+	d	d	+	+	+	+	+
L-Sorbose	d	d	-	+	+	+	+	-
Sucrose	+	d	+	+	+	+	+	+
D-Tagatose	d	-	-	d	-	d	d	d
D-Tartrate	d	-	-	-	-	(d)	d	-
Tricarallylate	d	-	-	+	+	-	-	+
Trigonelline	-	-	-	-	-	-	-	d
Tryptamine	-	-	-	-	-	-	-	d
D-Turanose	d	d	-	d	-	(d)	d	d
L-Tyrosine	-	-	-	-	-	-	-	d
D-Xylitol	d	-	-	(d)	-	d	d	d

Symbols and abbreviations: +, 95–100% strains positive in 1–2 days (utilization tests) or in 1 day (other tests); (+), 95–100% strains positive in 1–4 days; -, 95–100% strains negative in 4 days; d, different reactions; ND, no data ONPG, 2-nitrophenyl-β-D-galactopyranoside; and PQQ, pyrroloquinoline quinone.

Oxidation of glucose to gluconate (mediated by glucose dehydrogenase) in the absence of added pyrroloquinoline quinone is a unique property of *K. pneumoniae* subsp. *pneumoniae* among the nonmotile *Klebsiella* (Bouvet et al., 1989). This property is shared by *K. mobilis* (*E. aerogenes*). The other species require the addition of cofactor pyrroloquinoline quinone to express glucose dehydrogenase activity. Gluconate is oxidized to 2-ketogluconate (by a gluconate dehydrogenase) by all nutritionally versatile species except *K. oxytoca*.

Carbon source utilization tests are essential for the precise identification of *Klebsiella* species. Note that *K. oxytoca* is unique in producing a soluble yellow compound in all cupules showing some growth (Grimont et al., 1992b), and this may be the same pigment as that produced by this species on ferric gluconate (Ørskov, 1984a). Growth on *m*-coumarate never occurs with *K. terrigena* and *K. mobilis* (*E. aerogenes*). Growth on gentisate and 3-hydroxybenzoate never occurs with *K. pneumoniae* and *K. planticola*. Growth on histamine never occurs with *K. pneumoniae* and *K. oxytoca*. Growth on D-sorbose is always negative with *K. mobilis* (*E. aerogenes*), and growth on tricarballylate is always negative with *K. planticola*. Among the *Klebsiella*, only *K. terrigena* and some strains of *K. oxytoca* can grow on D-melezitose, and only *K. planticola* and *K. terrigena* can grow on 3-*O*-methyl-D-glucose. These eight carbon sources are often sufficient for the identification of all *Klebsiella* species (Monnet et al., 1991; Monnet and Freney, 1994). A multicenter evaluation showed that 18 biochemical tests allow identification of all *Klebsiella* species described so far (excluding *K. variicola*, which was not investigated; Hansen et al., 2004).

**IDENTIFICATION BASED ON MOLECULAR METHODS.** Identification of *Klebsiella* species and phylogenetic groups within *K. pneumoniae* and *K. oxytoca* can now be reliably achieved based on the sequencing of housekeeping genes such as *gyrA* and *parC* (Brisse and Verhoef, 2001) or *rpoB* (Drancourt et al., 2001). A simplified method based on *gyrA* PCR-RFLP was recently developed for species identification (Brisse et al., 2004b). Species can be identified by 16S rRNA gene sequencing, but because of limited nucleotide variation, 16S rRNA sequences cannot be reliably used to distinguish the phylogenetic groups (Brisse and Verhoef, 2001) within *K. pneumoniae* and *K. oxytoca* (S. Brisse, unpublished observation). *Klebsiella* species and phylogenetic groups can be identified using ribotyping (Brisse and Verhoef, 2001) or AFLP (Jonas et al., 2004). *Klebsiella oxytoca* can be differentiated from other *Klebsiella* species by a specific polymerase chain reaction (PCR) assay

targeting the *pehX* gene involved in pectin degradation (Kovtunovych et al., 2003).

## Ecology and Epidemiology

Ecological information obtained prior to the recognition of *K. oxytoca*, *K. planticola* or *K. terrigena* is certainly imprecise with respect to the identity of involved species, and mention of environmental isolates of *K. pneumoniae* should often read *Klebsiella* spp.

### *Klebsiella* in Water, Plants and Soil

*Klebsiella* spp. are often found in a variety of environmental sources such as soil, vegetation and water, contributing to biochemical and geochemical processes, and has been identified as a major component of the microflora in several types of stressed nonclinical environments (Huntley et al., 1976; Vasconcelos and Swartz, 1976; Seidler, 1981; Sjogren and Gibson, 1981; Niemelä et al., 1982). These bacteria have been recovered from aquatic environments receiving industrial wastewaters (Caplenas et al., 1981), plant products, fresh vegetables (Brown and Seidler, 1973), food with a high content of sugars and acids (Duncan and Razzell, 1972; Mundt et al., 1978), frozen orange juice concentrate (Fuentes et al., 1985), sugar cane wastes (Nunez and Colmer, 1968), living trees, plants and plant by-products (Brown and Seidler, 1973; Knittel et al., 1977; Caplenas et al., 1981).

They are commonly associated with wood (Duncan and Razzell, 1972), saw dust, and waters receiving industrial effluents from pulp and paper mills (Duncan and Razzell, 1972; Knittel, 1975; Dufour and Cabelli, 1976; Huntley et al., 1976) and textile finishing plants. As a consequence, the nasal cavities of many workers in paper and board mills proved to be contaminated by *Klebsiella* (Niemelä et al., 1985). *Klebsiella* spp. are also present in drinking water emanating from redwood tanks (Seidler et al., 1977). Isolates have been described several times in forest environments (Knittel et al., 1977; Bagley et al., 1978b), degrading kraft-lignin (Deschamps et al., 1980c), tannic acid (Deschamps et al., 1980b), pine bark (Deschamps and Lebeault, 1980a), and condensed tannins (Deschamps and Lebeault, 1980a; Deschamps and Lebeault, 1981), or from living or decaying wood and bark or composted wood (Deschamps et al., 1979; Deschamps et al., 1983).

*Klebsiella* strains from industrial effluents and a variety of human and bovine mastitis origins can multiply in pulp waste to levels exceeding 10<sup>6</sup> cells per ml. *Klebsiella* strains from vegetable surfaces or human infections could grow rapidly

on the surfaces of potatoes and lettuce to numbers exceeding  $10^3$  organisms per gram of surface peel and leaf after 24 h incubation at room temperature (Knittel et al., 1977).

*Klebsiella* can frequently be isolated from the root surfaces of various plants (Pedersen, 1978; Haahtela et al., 1981). *Klebsiella pneumoniae*, *K. oxytoca* or *K. planticola* can fix nitrogen and are classified as associative nitrogen fixers, or diazotrophs (Mahl et al., 1965; Ladha et al., 1983). Isolation of nitrogen-fixing *Klebsiella* was reported from rice leaves (Thomas-Bauzon et al., 1982; Ladha et al., 1983), rhizosphere (Rennie, 1982), grassland soil (Line and Loutit, 1971), decaying wood (Seidler et al., 1972), paper mill liquors and effluents (Nielson and Sparell, 1976), and maize stem tissue (Palus et al., 1996). The endophytic lifestyle of two *K. pneumoniae* strains was demonstrated using green-fluorescent protein labeling (Chelius and Triplett, 2000). These strains colonized the intercortical layers of the stem and the maturation region of the root. Association of *K. oxytoca* with barley rhizosphere during an entire vegetative period was demonstrated using a bioluminescence reporter plasmid (Kozyrovska et al., 1994).

Purified type 3 fimbriae, as well as type 1 fimbriae (see the section Properties Relevant to Pathogenicity for Humans in this Volume) of nitrogen-fixing *Klebsiella*, mediate bacterial adhesion to plant roots (Korhonen et al., 1983; Haahtela et al., 1985b). However, type 3 fimbriae are more efficient than type 1 fimbriae in promoting adherence (Haahtela and Korhonen, 1985a). The bacteria adhere strongly to root hairs, and with a markedly lower efficiency, to the surface of the zone of elongation and to the root cap mucilage (Haahtela et al., 1986). No adhesion to the epidermal cell between root hairs was observed (Haahtela et al., 1986).

A number of the *Klebsiella* strains reported from plant material might be *K. planticola* (Bagley et al., 1981). Strains of *K. pneumoniae* sensu stricto, which are associated with plants (e.g., living or decaying wood and bark), differ from those associated with serious human infections. These environmental *K. pneumoniae* strains are most often able to utilize 5-ketogluconate as sole carbon source (P.A.D. Grimont, unpublished observation) and never have capsular types K1 to K6. Strains involved in serious infection do not utilize 5-ketogluconate and may have capsular types K1 to K6, as well as other capsular types. A comparison of three endophytic *K. pneumoniae* strains with two clinical strains (Dong et al., 2003) showed that the plant-associated strains belong to KpIII (Brisse and Verhoef, 2001), whereas the two clinical strains belong to KpI and were not able to colonize the interior of wheat seedlings as efficiently as the

endophytic strains. However, note that KpIII strains may be found in clinical samples from hospitalized patients (Brisse and Verhoef, 2001).

### *Klebsiella* in Animals

*Klebsiella* spp. can be found in a wide range of mammals (Gordon and FitzGibbon, 1999) and are also present in insects (Dillon et al., 2002) and possibly in many other animal groups. In locusts, *Klebsiella* may contribute, among other bacterial species, to the synthesis of the aggregation pheromone implicated in the change from a grasshopper-like solitary insect to the gregarious form (Dillon et al., 2002). Cockroaches were suggested to play the role of vectors in the hospital environment (Cotton et al., 2000).

*Klebsiella pneumoniae* is an important cause of metritis in mares. Capsular types K1, K2, and K5 were major causes of epidemic metritis in England, whereas type K7 was associated with sporadic, opportunistic genital infection (Platt et al., 1976). Outbreaks of metritis in mares were due to type K2 in the United States and France (Edwards, 1928; Tainturier and Richard, 1986). The stallion plays an important role in the transmission of *K. pneumoniae*. Type K7 was found on the preputial skin of stallions and may be part of the normal bacterial flora in this location (Platt et al., 1976). Thus it is important to determine the K-type of *Klebsiella* strains isolated from the genital tract of horses to detect stallion carriage of an epidemic strain versus carriage of less pathogenic *K. pneumoniae*.

*Klebsiella* have been frequently associated with bovine mastitis (Braman et al., 1973) and causes serious infections in other animals including dogs, Rhesus monkeys, guinea pigs, muskrats and birds (Wyand and Hayden, 1973; Fox and Rohovsky, 1975; Kinkler et al., 1976; Wilson, 1994; Roberts et al., 2000). Epidemics of fatal generalized infections among captive squirrel monkeys (*Saimiri sciureus*) in French Guyana and lemurs in a French zoo were due to *K. pneumoniae* K5 and K2, respectively (Richard, 1989). Immunization of the monkeys (or lemurs) with the corresponding capsular polysaccharide was efficient in stopping the epidemic (Postal et al., 1988; Richard, 1989).

### *Klebsiella* in Humans

The interactions of *Klebsiella* with humans range from asymptomatic carriage to opportunistic infections (mainly in hospitalized patients), and also include community-acquired infections. Most *Klebsiella* infections now occur in hospitalized patients and many are caused by antibiotic-resistant strains, including strains that produce extended-spectrum  $\beta$ -lactamases (ESBLs) that



are a serious cause of concern. The importance of klebsiellae as nosocomial pathogens, their epidemiology, and their pathogenicity were reviewed by Podschun and Ullman (1998a).

*Klebsiella pneumoniae* was sometimes called “Friedländer’s bacillus” or the agent of Friedländer’s pneumoniae, a community-acquired pulmonary infection that was observed in chronic alcoholics (Carpenter, 1990) and had characteristic radiographic features that were due to severe pyogenic infection. Not all capsular types are associated with acute pneumonia. Type K1 is most frequently isolated in these situations, but K2, K3, K4, K5, and K6 can also be involved (Eickoff et al., 1966; Ørskov, 1981). Recent data on the prevalence of community-acquired *Klebsiella pneumoniae* are not abundant, but Friedländer’s pneumoniae appears to have become rare in the Western world (Vergis et al., 2000). In a multicenter study, Ko et al. (2002) found only four cases of community-acquired bacteremic *K. pneumoniae* pneumonia in hospitals in Europe, the United States, Argentina and Australia, whereas 53 such cases were observed in South Africa and Taiwan. Other reports also indicate that community-acquired *Klebsiella pneumoniae* is more frequent in South Africa and in Asian countries, where it sometimes ranks first among etiological agents of community-acquired pneumonia (Potgieter and Hammond, 1992; Feldman et al., 1995; Jong et al., 1995; Ishida et al., 1998; Liam et al., 2001).

In humans, carriage is frequent in the intestinal tract and also occurs, albeit less frequently, in the nasopharynx. The number of *Klebsiella* bacteria can attain  $10^7$  per gram of dry feces (Finegold et al., 1983; Leclerc et al., 2001). *Klebsiella* spp. are found in fecal material from a considerable proportion of persons (Thom, 1970; Montgomerie, 1979; de la Torre et al., 1985; MacFarlane and Narla, 1985). A survey of *Klebsiella* carriage in urban residents (not associated with a hospital environment), hospital personnel, and newly admitted patients showed 30–37% of individuals carry *Klebsiella* (various serotypes), including 29–35% fecal carriage and 3–4% throat carriage (Davis and Matsen, 1974). There was a slight increase in prevalence among long-term patients (43% carriage). Note that about 34% of the strains in this survey were indole-positive (Davis and Matsen, 1974). In general, patient colonization by *Klebsiella* spp. increases with length of stay and antibiotic use (Rose and Schreier, 1968; Pollack et al., 1972; Podschun and Ullmann, 1998a). Health-care workers can also have high rates of *Klebsiella* carriage (Casewell and Talsania, 1979; Cooke et al., 1979). Carriage is considered the major reservoir of *Klebsiella* infections (Selden et al., 1971; Montgomerie, 1979; Podschun and

Ullmann, 1998a). Epidemiological markers (e.g., capsular typing) are necessary to understand the origin of infection (see the section Typing Methods in this Chapter). The endogenous origin of an infection is often claimed because the same capsular type is found in the infected site and in the gut of the patient. This should not mean that no prevention is feasible since the intestinal *Klebsiella* may have been acquired during the hospital stay.

Features predisposing to *K. pneumoniae* or *K. oxytoca* nosocomial infection include extremes of age, chronic alcoholism, diabetes mellitus, chronic cardiac, renal, and pulmonary and neoplastic disease (Feldman et al., 1990; Hansen et al., 1998).

Infections with *Klebsiella* are caused mainly by *K. pneumoniae* and *K. oxytoca* in a proportion estimated at 2 to 1 (Bauernfeind et al., 1981) or 4 to 1 (de la Torre et al., 1985). Most *Klebsiella pneumoniae* infections are caused by strains of phylogenetic group KpI, but groups KpII and KpIII (which corresponds to *K. variicola*) are also found (Brisse et al., 2004b). Infections with *K. planticola* and *K. terrigena* have long been considered exceptional, but this may be due in part to the difficulties of their identification (Monnet et al., 1991; Monnet and Frenay, 1994). In fact, recent reports show that *K. planticola*, and to a lesser extent *K. terrigena*, are not rare in clinical infections (Mori et al., 1989; Podschun and Ullmann, 1992a; Podschun et al., 1998b; Westbrook et al., 2000; Stock and Wiedemann, 2001). *Klebsiella planticola* has been isolated from newborns (Hart, 1993; Podschun et al., 1998b; Westbrook et al., 2000). *Klebsiella planticola* and *K. ornithinolytica* have also been directly involved in histamine fish poisoning (Kanki et al., 2002; see below).

Klebsiellae are widely recognized as important opportunistic pathogens in hospital patients, representing 3–8% of all nosocomial bacterial infections and ranking second (behind *Escherichia coli*) as a cause of nosocomial Gram-negative bacteremia (Podschun and Ullmann, 1998a). They are mostly associated with infections of the urinary and respiratory tracts, as well as wound and soft tissue infections, and can cause fatal septicemia. *Klebsiella* strains have the ability to spread extensively among patients, leading to nosocomial outbreaks, especially in neonatal units (Hart, 1993; Podschun and Ullmann, 1998a). Ørskov (1952) first described clusters of urinary tract infections due to *K. pneumoniae* among hospitalized patients, followed by innumerable numbers of authors who described nosocomial outbreaks of *Klebsiella* strains, especially multiresistant strains (for references, see the section Typing Methods in this Chapter). Plasmids encoding ESBLs carried by *Klebsiella* strains can

also spread to other bacterial strains and species (Coque et al., 2002). Interhospital transmission of *Klebsiella* strains, mostly those producing ESBLs, have been described (Buré, 1988; Arlet et al., 1994; Monnet et al., 1997). Capsular types of *Klebsiella* strains found in hospitals are diverse (Hansen et al., 1998).

In addition to the above-mentioned prevalence of community-acquired *K. pneumoniae* pneumonia in Asia, three severe clinical manifestations have emerged as important public health problems in this part of the world. First, primary bacteremic liver abscess has been described since 1989 in Asia, especially Taiwan (Chang and Chou, 1995; Ko et al., 2002). The Taiwanese patients have no history of hepatobiliary disease, but 70% have diabetes mellitus (Ko et al., 2002). Second, *K. pneumoniae* endophthalmitis, also almost exclusively reported from Asia, may represent a secondary complication of liver abscess (Hidaka et al., 1993; Ko et al. [2002] and references therein). Third, *K. pneumoniae* is the cause of community-acquired bacterial meningitis in adults in Taiwan, even with no association with liver abscess or infections of other body sites. The proportion of cases of culture-proven *K. pneumoniae* bacterial meningitis in one Taiwanese hospital increased from 8% (between 1981 and 1986) to 18% (between 1987 and 1995; Tang et al., 1997; Ko et al., 2002). The genetic basis of these peculiar pathological aspects is so far unknown. The capsular serotypes of blood isolates found in one Taiwan hospital were diverse, although K1 predominated (Cheng et al., 2002). Ribotyping and pulse-field gel electrophoresis (PFGE) also revealed genetic variation among the strains, suggesting that liver abscess is not caused by a single clone. The complication of endophthalmitis, however, may be associated with capsular serotype K1 (Fung et al., 2002).

Recent studies have associated *K. pneumoniae* with chronic diarrhea in HIV-infected persons (Gassama et al., 2001; Nguyen Thi et al., 2003). These seemingly enteropathogenic strains display aggregative adherence to HEp-2 cells, but they do not appear to harbor virulence factors known to be involved in pathogenicity of pathogenic *E. coli* strains (Nguyen Thi et al., 2003).

*Klebsiella pneumoniae* has been implicated in the development of ankylosing spondylitis because of the high incidence of *Klebsiella* in the bowel flora of patients whose disease is in an active state (Ebringer et al., 1979; Calin, 1984). It is striking that 70–90% of the affected patients share HLA type HLA-B27 (a phenotype present in less than 10% Caucasians). The mimicry between *K. pneumoniae* K43 and human HLA-B27 antigens might be responsible for the development of arthritis (Ogasawara et al., 1986).

*Klebsiella pneumoniae* subsp. *rhinoscleromatis* is classically associated with rhinoscleroma, a chronic granulomatous disease of the nose. This disease is endemic in Africa, eastern and central Europe, and South and Central America (Ørskov, 1981; Richard, 1982; Hart and Rao, 2000).

*Klebsiella pneumoniae* subsp. *ozaenae* is considered to be associated with ozena, an atrophic rhinitis with an unpleasant smell (Malowany et al., 1972; Shehata, 1996; Chand and MacArthur, 1997). In the past 10 years, several reports have stated that *K. ozaenae* (now *K. pneumoniae* subsp. *ozaenae*) may cause invasive infections, especially in immunosuppressed hosts: bacteremia with or without meningitis (Berger et al., 1977; Goldstein et al., 1978; Lewis and Alexander, 1979; Murray et al., 1981), otitis, mastoiditis, urinary tract infections, wound infections, corneal ulcers, pneumonia (Berger et al., 1977; Goldstein et al., 1978; Murray et al., 1981; McCarthy and Hubbard, 1984; Janda et al., 1985), or brain abscess (Strampfer et al., 1987). In most of these cases, however, *K. pneumoniae* subsp. *ozaenae* was identified with the sole help of commercial identification kits without confirmation of the identification by capsular typing (see the section Identification in this Chapter).

*Klebsiella granulomatis* (formerly *Calymmatobacterium granulomatis*) is the presumed causative agent of granuloma inguinale (donovanosis), a genital ulceration that is sexually transmissible. Histologic features include dense dermal infiltrate of plasmacytes, neutrophils and large macrophages with vacuolated cytoplasm that contain intracellular bacilli (Donovan bodies). Carter et al. (1999) pointed out the similarity of donovanosis and rhinoscleroma histologic lesions, which parallels the close genetic relationship of both species to *K. pneumoniae*, but whether the pathogenic properties of these species have a common evolutionary origin is yet unknown.

## Properties Relevant to Pathogenicity for Humans

The pathogenicity of *Klebsiella* has been reviewed by Williams and Tomas (1990) and Podschun and Ullmann (1998a). Four components of *Klebsiella* have long been implicated in pathogenesis: adhesins, capsular polysaccharides, lipopolysaccharide (LPS), and iron-scavenging systems (siderophores). Recently, systematic attempts to identify new components involved in colonization and infection have been made using signature-tagged mutagenesis (Maroncle et al., 2002; Struve et al., 2003b), genomic subtractive hybridization (Lai et al., 2000), in vivo expression

technology (Lai et al., 2001), and transposon mutagenesis (Cortes et al., 2002a). Genes possibly involved in *Klebsiella* pathogenesis were identified in these studies, but characterization of their function and role in *Klebsiella* virulence has only just begun (Cortes et al., 2002b; Lai et al., 2003a). Although *K. pneumoniae* is considered an extracellular pathogen, invasion of cultured human epithelial cells from the bladder and ileocecum by *K. pneumoniae* was reported (Fumagalli et al., 1997; Oelschlaeger and Tall, 1997), and it was shown that capsule interferes with adhesion and invasion (Sahly et al., 2000).

### Adhesins

Adhesion to mucosal and epithelial cell surfaces is often the first step in the development of colonization and infection. Adhesins are often also hemagglutinins and may be located on fimbriae that protrude on the surface of the bacterial cells. *Klebsiella* fimbriae were studied by Duguid (1959), who demonstrated their association with different hemagglutination (HA) behaviors. Strains of *K. pneumoniae*, *K. oxytoca*, *K. planticola* and *K. terrigena* may produce thick, channelled (type-1) fimbriae, which are closely related to type 1 fimbriae produced by other Enterobacteriaceae species. *Klebsiella* type 1 fimbriae are responsible for D-mannose-sensitive hemagglutination (MS-HA) and cause agglutination of guinea pig erythrocytes. More than 80% of clinical isolates of *K. pneumoniae*, but few *K. oxytoca* strains, express type-1 fimbriae (Przondo-Hessek, 1983; Podschun and Sahly, 1991). Clinical and fecal carriage isolates of *K. pneumoniae* express type 1 fimbriae in higher proportion than environmental strains (Podschun and Sahly, 1991). *Klebsiella mobilis* (*E. aerogenes*) produces antigenically similar fimbriae, which are not associated with hemagglutinin activity. *Klebsiella* type 1 fimbriae are antigenically distinct from those of *Enterobacter* species (Adegbola and Old, 1985). A gene cluster has been found to contain several genes required for the synthesis and assembly of type 1 fimbriae and a 29-kDa protein, the adhesin, located at the tip and at intervals along the fimbria (Purcell et al., 1987; Abraham et al., 1988). At least two forms of the gene, which are antigenically distinct, exist in *K. pneumoniae* (Gerlach et al., 1989a). Type 1 fimbriae mediate the attachment of *K. pneumoniae* to uroepithelial cells and are involved in the development of bladder infection in the rat (Fader et al., 1988; Williams and Thomas, 1990). These fimbriae also mediate adherence to ciliated tracheal cells in vitro (Fader et al., 1988). Although they are believed to be primarily involved in the pathogenesis of lower urinary tract infections, type 1 fimbriae may be involved

in pyelonephritis (Fader and Davis, 1982). The expression of type 1 fimbriae is subjected to phase variation and may be switched off (Fader and Davis, 1982), which is believed to be advantageous to the bacteria in the latter stages of the infection, as these fimbriae can be recognized by leukocytes and trigger the destruction of the bacteria through lectinophagocytosis (Ofek et al., 1995).

Strains of *Klebsiella* also produce thin, non-channelled (type-3) fimbriae that agglutinate ox erythrocytes only when these have been previously treated with tannin, in the absence or presence of D-mannose. This agglutination type has been called "mannose-resistant, *Klebsiella*-like hemagglutination" (MR/K-HA) because it was initially discovered in *Klebsiella* strains, but it is found in many other species (Clegg and Gerlach, 1987). Przondo-Hessek and Pulverer (1983) found that 85% of *Klebsiella* clinical isolates possess the mannose-resistant *Klebsiella* hemagglutinin, mediated by type 3 fimbriae. Podschun and Sahly (1991) found that most strains of *K. pneumoniae* and *K. oxytoca* induced MR/K agglutination. In both species, MR/K agglutination was significantly more frequent in clinical and fecal carriage isolates than in sewage isolates (Podschun and Sahly, 1991).

Type 3 fimbriae can mediate binding to plant roots (Korhonen et al., 1983; Haahtela et al., 1985b), human endothelial cells, epithelia of the respiratory tract, uroepithelial cells, and type V collagen (Tarkkanen et al., 1990; Wurker et al., 1990; Hornick et al., 1992; Tarkkanen et al., 1997). MR/K-HA is inhibited by the polyamine spermidine (Gerlach, 1989b). Spermidine has been suggested to act as a receptor of type 3 fimbriae, as it is present on the surface of damaged erythrocytes but not on untreated ones (Gerlach, 1989b). However, the role of type 3 fimbriae in human infection has not been directly established (Podschun and Ullmann, 1998a).

Type 3 fimbriae are encoded by the *mrk* gene cluster (Allen et al., 1991). The fimbriae are made of a fimbrial shaft composed predominantly of the major subunit MrkA and of the MrkD adhesin that is assembled into the shaft. Functional heterologous fimbriae can be assembled by expressing the *mrkD* gene in a strain containing the *pap* gene cluster (Hornick et al., 1995), suggesting that the assembly apparatus for type 3 fimbriae is related to that of P pili of uropathogenic *E. coli*. In *Klebsiella*, the *mrkD* gene shows variation among hemagglutinating strains (Schurtz et al., 1994). A form of the *mrkD* gene was found on a large native plasmid (Hornick et al., 1995). This form, *mrkD*<sub>1P</sub>, is frequently found on plasmids among *K. oxytoca* strains, but not among *K. pneumoniae* strains, which have a distinct chromosomal gene,

*mrkD*<sub>1C</sub>. The other *mrk* genes of the cluster are more conserved, regardless of whether they are present on a chromosome or a plasmid (Hornick et al., 1991; Hornick et al., 1995). Type V collagen-binding specificity is a function of the MrkD<sub>1P</sub> protein (Tarkkanen et al., 1990; Hornick et al., 1995), whereas the presence of MrkD<sub>1C</sub> is associated with the ability to bind both type IV and type V collagen. However, some hemagglutinating strains possessing a gene related to *MrkD*<sub>1C</sub> do not bind to either type IV or type V collagen (Sebghati et al., 1998). Mutations impairing either assembly into the fimbrial shaft or binding to type V collagen were identified (Sebghati and Clegg, 1999).

Type 3 fimbriae could be responsible for colonization of the surfaces of indwelling devices, as they are involved in adherence to abiotic surfaces and biofilm formation (Langstraat et al., 2001; Di Martino et al., 2003). Biofilm formation appears to be mediated by the fimbrial shaft protein MrkA and not by the adhesin (Langstraat et al., 2001).

Other adhesins have been described in *Klebsiella*. In a search for bacterial factors responsible for the adhesion of *K. pneumoniae* to intestinal cells, Darfeuille-Michaud et al. (1992) found a nonfimbrial adhesin of 29 kDa that was designated "CF29K." Its gene was encoded on a large conjugative plasmid, which also contained genes for broad-spectrum cephalosporin resistance and aerobactin (Darfeuille-Michaud et al., 1992). A novel fimbrial adhesin termed "KPF-28" was found (Di Martino et al., 1996) as a long, thin, and flexible fimbria 4–5 nm in diameter and 0.5–2 µm long. It is involved in the adherence to the human carcinoma cell line Caco-2. As for CF29K, this adhesin was also encoded on a plasmid, and it was associated to strains producing SHV-4 extended-spectrum β-lactamase. ("SHV" is a contraction of "sulfhydryl variable": a description of the biochemical properties of this β-lactamase.)

The role of the capsular polysaccharide in promoting or impairing adhesion to epithelial cells and mucosal surfaces has been recently re-evaluated. While capsular polysaccharide may enhance adhesion to mucus-producing cells and appears necessary for colonization of large intestines of streptomycin-treated mice (Favre-Bonte et al., 1999a; Favre-Bonte et al., 1999b), it impedes the adhesion to epithelial cells (Favre-Bonte et al., 1999a; Sahly et al., 2000; Struve and Kroghelt, 2003a). Capsular-like material has also been suspected to be responsible for aggregative adherence to human intestine-407 cells (Favre-Bonte et al., 1995). The capsule may interfere with the expression of adhesins, perhaps at the transcriptional level (Favre-Bonte et al., 1999a; Sahly et al., 2000).

## Capsular Polysaccharides

*Klebsiella* strains are surrounded by a generally thick hydrophilic polysaccharide capsule responsible for the glistening, mucoid aspect of colonies on agar plates. A total of 77 antigenically distinct exopolysaccharides have been recognized so far and are included in the international K-serotyping scheme (Ørskov and Fife-Asbury, 1977). The chemical structure of most K-antigen types has been analyzed (Ørskov and Ørskov, 1984b). Capsular polysaccharides are acidic and composed of repeats of basic units of four to six sugars. It has been shown that noncarbohydrate groups are also present on some capsular types, but very little is known about the immunochemical specificity of *Klebsiella* capsular polysaccharides.

The capsule was the first virulence factor described for *Klebsiella* (Toenniessen, 1914; Baerthlein, 1918; Kauffmann, 1949; Mizuta et al., 1983; Williams et al., 1983; Cryz et al., 1984a). In 1928, Edwards showed that capsule production by *Klebsiella* was necessary to produce metritis in mares (Edwards, 1928). A relationship was found between the size of capsule (capsular types 1 and 2) and pathogenicity in diverse experimental models (Domenico, 1982; Cryz et al., 1984a). Pollack (1976) demonstrated detectable levels of circulating capsular polysaccharide in patients infected by *K. pneumoniae* and suggested a correlation between antigenemia and severity of infection. The increased virulence conferred by the capsular material has been explained on the basis of protection against phagocytosis by polymorphonuclear leukocytes (Simoons-Smit, 1984; Podschun et al., 1992; Podschun and Ullmann, 1992b) and against killing by serum (Williams et al., 1983; Simoons-Smit et al., 1986). The K-antigen plays a crucial role in protecting the bacterium from opsonophagocytosis in the absence of specific antibodies, by blocking complement deposition or activation (Williams et al., 1983; Simoons-Smit et al., 1986; Williams et al., 1986; Williams and Thomas, 1990). Capsular polysaccharide was also shown to be essential to *K. pneumoniae* virulence in a murine model of pneumonia (Cortes et al., 2002a). In this model, the capsule modulates C3 deposition and protects the bacteria against alveolar macrophage phagocytosis.

Differences in virulence between *Klebsiella* capsular serotypes have long been recognized (Kauffmann, 1949). On the basis of mouse lethality, Mizuta et al. (1983) showed that serotypes K1 and K2 were the most virulent *Klebsiella* serotypes when injected intraperitoneally. However, the finding of avirulent strains belonging to the O1:K2 serogroup that were fully encapsulated, suggested that factors other than

the cell-associated capsular material could play a role in virulence. Simoons-Smit et al. (1984) examined the virulence of clinical isolates of *Klebsiella* in a mouse-skin model and showed that strains with K1, K2, K4, and K5 capsular antigens were more virulent for mice than strains with K6 and K above 6. Note, however, that not all K serotypes have been tested in these studies.

The mannose content of the capsular polysaccharide may determine the degree of virulence associated with a given K serotype. Capsular polysaccharides of low-virulence serotypes, such as K7 and K21a (Podschun et al., 1992c; Ofek et al., 1993), have specific mannose-containing structures and are recognized by a lectin on the surface of the macrophages. Through the phenomenon termed "lectinophagocytosis" (Athamna et al., 1991), macrophages ingest and kill the bacteria harboring these structures. In contrast, K2 polysaccharides, which do contain mannose but in a different configuration, are not recognized by macrophages (Ofek et al., 1993; Kabha et al., 1995). In elegant experiments of genetic exchange of the capsular polysaccharide gene clusters between a K2 strain and a K21a strain, Ofek et al. (1993) showed that the recipient strain expressing heterologous *cps* genes exhibited the macrophage-binding phenotype of the donor. In another study, this group reported that surfactant protein A, the main protein component of lung surfactant, enhances the phagocytosis by alveolar macrophages of *Klebsiella* K21a strains but not of K2 strains, a reaction that was inhibited by mannan (Kabha et al., 1997). Zamze et al. (2002) found that the macrophage mannose receptor did not bind to the capsular polysaccharides of a number of *Klebsiella* K-types. In this study, no direct correlation was found between the structure of the polysaccharide and binding to the mannose receptor, suggesting that conformation, rather than sugar content, may be important. However, serotypes K2, K7 and K21 were not tested in this study (Zamze et al., 2002).

Studies of the distribution of K-serotypes among *Klebsiella* clinical isolates showed very distinct prevalence patterns of K-types across distinct geographic regions (Richard, 1973; Rennie and Duncan, 1974; Blanchette and Rubin, 1980; Simoons-Smit et al., 1985; Cryz et al., 1986; Toivanen et al., 1999; Fung et al., 2000). For example, serotype K1 is frequently isolated in studies from Taiwan, China and Japan but is absent from most seroepidemiology studies in Europe and the United States. K2 and K21 appear to be the only frequently found serotypes in most studies (Fung et al., 2000), but other studies reported very few K2 strains (Rennie and Duncan, 1974; Simoons-Smit et al., 1985). The reasons for these differences are not understood and may include

geographic differences, temporal shifts linked to antibiotic usage or other selective pressures, and inconsistent identification of *Klebsiella* species combined with distinct prevalence of K-types among species. However, knowledge of the prevalence of K-types is relevant to vaccine development (Cryz et al., 1985) or humoral immunotherapy (Donta et al., 1996; Lepper et al., 2003) based on capsular polysaccharides.

The genomic organization of the chromosomal region that is responsible for capsular polysaccharide synthesis in a K2 *K. pneumoniae* strain was determined by Arakawa et al. (1995). Nineteen possible open reading frames (ORFs) were identified in the 24,329-bp nucleotide sequence of the *cps* region. Most of them appear to be organized into two transcriptional units. Among these ORFs, the *gnd* gene coding for 6-phosphogluconate dehydrogenase was identified downstream of the longest transcriptional unit. This gene separates the bulk of *cps* genes from two genes responsible for the synthesis of the mannose residue that is part of the K2 polysaccharide unit (Arakawa et al., 1995). The genomic organization of the *cps* region has not been described for other *Klebsiella* K antigens. Rahn et al. (1999) have pointed out the conservation of the genetic organization of the *cps* gene cluster between some *Klebsiella* serotypes and some *E. coli* group 1 K antigens. *Escherichia coli* K30 and *Klebsiella* K20 antigens have a high degree of similarity in gene organization and nucleotide sequence. In these strains, the sequence upstream of the *cps* cluster shows the presence of insertion sequence (IS) elements, which may have been responsible for the horizontal transfer of the *cps* locus between these species (Rahn et al., 1999).

Some encapsulated *K. pneumoniae* isolates form glistening mucoid colonies of viscid consistency. Loss of this phenotype in subcultures was associated with a reduction of virulence (Takahashi et al., 1977). This mucoid phenotype was earlier considered to be the result of overproduction of capsular polysaccharide (Takahashi et al., 1977). Nassif et al. demonstrated that the mucoid phenotype is determined by a gene, designated "*rmpA*" (for regulator of mucoid phenotype), located on a 180-kb plasmid, which also carries the aerobactin gene (Nassif et al., 1989a; Nassif et al., 1989b). A mutation in *rmpA* increases the 50% lethal dose for mice by 1000-fold, although the mutated strain still expresses its capsular specificity. When this gene is introduced in *E. coli*, it triggers the production of colanic acid. Hypothetically, *rmpA* is a regulatory gene which controls the production of a shield (of unknown composition) surrounding the bacteria and protecting them from interaction with anticapsule-specific antibodies

(Nassif et al., 1989a; Nassif et al., 1989b). Similar findings were reported by Wacharotayankun et al. (1993) from another K2 *K. pneumoniae* strain, CG43, in which the about 200-kb plasmid was called “pLVKP” (for large virulence plasmid of *Klebsiella*), and the gene that enhances the mucoidy of colonies was called “*rmpA2*.” RmpA2 was shown to function as a trans-acting regulator for the capsule synthesis (Arakawa et al., 1991; Wacharotayankun et al., 1992) and to regulate K2 capsule expression at the transcriptional level (Lai et al., 2003b). Note that the two-component regulatory system genes, *rcaA* and *rcaB* (for regulator of capsule synthesis), have been cloned from *K. pneumoniae* and shown to activate colanic acid production when expressed in *E. coli* (Allen et al., 1987; McCallum and Whitfield, 1991). Therefore, it is believed that RmpA (or RmpA2) increases capsule production above that normally achieved by Rcs activation alone (Rahn and Whitfield, 2003).

### Lipopolysaccharide

The lipopolysaccharide (LPS) molecule is composed of lipid A, a core polysaccharide and a side chain called the “O-antigen.” Nine O-antigen types are distinguished in *K. pneumoniae*, O1 being the most frequent (Hansen et al., 1999). The most important role of the O-antigen is to protect *K. pneumoniae* from complement-mediated killing, as capsulate or non-capsulate strains lacking the O1 antigen are very sensitive to the bactericidal action of both the alternative and classical complement pathways (Williams et al., 1983; Tomas et al., 1986; McCallum et al., 1989; Williams and Thomas, 1990). For this protection, O-antigen chain length seems to be important (Ciurana and Tomas, 1987; McCallum et al., 1989). C3b is readily deposited on the surface of K<sup>-</sup> O<sup>+</sup> cells, but the membrane attack complex fails to kill the cells, possibly due to a steric effect of the long O chain (Williams and Thomas, 1990). Nevertheless, the O-antigen is extremely efficient at activating the early components of the complement, and opsonization renders K<sup>-</sup> O<sup>+</sup> bacteria susceptible to phagocytosis in nonimmune serum (Williams and Thomas, 1990). These results have been obtained for the O1 antigen, and it is unclear whether they can be extended to other O-types. However, using well-characterized isogenic mutants, Merino et al. (2000) showed that the O5-antigen LPS is also essential for serum resistance and is an important factor for the adhesion to uroepithelial cells, possibly because of its surface charge and hydrophobicity properties. In contrast, Cortes et al. (2002a) showed that the LPS plays no major role in the establishment of *K. pneumoniae* pneumonia in a murine model.

The O1 lipopolysaccharide has been linked with the extensive tissue necrosis that complicates *Klebsiella* infections (Straus et al., 1985). The production of an extracellular toxic complex (ETC) that has been shown to be responsible in mice for lethality and extensive lung necrosis is composed of 63% capsular polysaccharide, 30% lipopolysaccharide, and 7% protein (Straus, 1987). Antibodies produced against the lipopolysaccharide portion (possessing the toxicity) of the ETC have been shown to be protective (Straus, 1987).

The cluster of genes responsible for the synthesis of the core lipopolysaccharide of *K. pneumoniae* has been genetically characterized (Noah et al., 2001; Regue et al., 2001). Disruption of one of the ORFs, *orf10*, resulted in a two-log-fold reduction of virulence in mice, as well as in a strong reduction in the capsule amount (Regue et al., 2001). A monoclonal antibody that is specific for the genus *Klebsiella* was identified that binds to a conserved epitope of the core region (Brade et al., 2001).

### Siderophores

Iron is essential for bacterial growth. Brewer et al. (1982) observed that virulence was enhanced by hyperferremia, suggesting that iron scavenging may also be a virulence factor. In the human body, iron is complexed to carrier molecules such as transferrin (in the serum) or lactoferrin (in milk and other secretions), or sequestered within cells (in heme proteins). Under conditions of iron limitation, potentially pathogenic Enterobacteriaceae produce high-affinity systems (siderophores) to solubilize and import the required iron. The iron-chelating compounds produced are mostly of two sorts, phenolates (e.g., enterochelin) and hydroxamates (aerobactin; Payne, 1988). In response to iron deprivation, *K. pneumoniae* strains were found to induce between four and six iron-repressible outer-membrane proteins in the 70–85 kDa range. All strains were found to produce enterochelin, while only a few could produce aerobactin (Williams et al., 1987). Krone et al. (1985) showed that *K. pneumoniae* strains belonging to serotype K2 harbor a large conjugative plasmid that encodes production of aerobactin and a 76-kDa outer membrane protein. Nassif and Sansonetti (1986) correlated the virulence of *K. pneumoniae* serotypes K1 or K2 with the presence of a 180-kb plasmid encoding the hydroxamate siderophore aerobactin (in addition to gene *rmpA*, see above). The introduction of the aerobactin genes within an avirulent plasmidless recipient enhanced the virulence by 100-fold, but virulence did not reach the level of the wild-type strain. These results suggested that aerobactin is an essential factor of pathogenicity

(Nassif, 1986). Some strains of *K. pneumoniae* express a ferric aerobactin uptake system without making the chelator itself. This may confer a selective advantage in mixed infections in competition with other aerobactin-producing bacteria (Williams and Thomas, 1990). A third siderophore called “yersiniabactin,” which is encoded by the *Yersinia* high-pathogenicity island, was shown to be present in *Klebsiella* (Bach et al., 2000; Koczura and Kaznowski, 2003), but its prevalence in *Klebsiella* species and its role in *Klebsiella* pathogenesis are unknown.

### Other Factors

Little is known about the hemolysins produced by *Klebsiella* that were considered as non-hemolytic for human red blood cells (Smith and Ngui-Yen, 1980) and found to be hemolytic in rabbit blood agar (Albesa et al., 1980). Two hemolysins active on rabbit red blood cells were purified by Albesa et al. (1985), who gave the first description of a thiol-activated hemolysin in Gram-negative bacteria. Camprubi et al. (1990) reported that a high level of hemolysin production and excretion could be influenced by the presence of lipopolysaccharide.

Other potential virulence factors produced by *K. pneumoniae* possibly include the production of heat-labile and heat-stable endotoxins (Wadstrom et al., 1976; Klipstein et al., 1983). Recent factors undergoing evaluation for a role in pathogenesis include the two-component system KvgAS (Lai et al., 2003a), HtrA (which may be involved in serum complement resistance; Cortes et al., 2002b), a protein-tyrosine kinase, and a phosphotyrosine-protein phosphatase (the last two being involved in the synthesis of capsular polysaccharide; Preneta et al., 2002).

### Human Vaccine

An alternative to the use of antibiotics is to attempt immunological control of *Klebsiella* infections, either by vaccination of patients at risk or by immunotherapy. Anti-capsular polysaccharide antibodies were found to provide a high degree of protection against corresponding capsular serotypes (Cryz et al., 1984b; Cryz et al., 1985). A 24-valent *Klebsiella* capsular polysaccharide vaccine has been prepared by Cryz et al. (1985). Phase I trials indicated that the vaccine was safe (Edelman et al., 1994). A good antibody response was observed with this vaccine in patient victims of acute trauma, with nine out of ten patients responding to at least 18 of the 24 antigens (Campbell et al., 1996). The 24-valent vaccine was used to immunize 2000 healthy volunteers (simultaneously with a *Pseudomonas* vaccine) to obtain a hyperimmune

intravenous immunoglobulin preparation, which was tested in a randomized clinical trial in intensive care unit patients (Donta et al., 1996). The incidence and severity of infections caused by *Klebsiella* strains with K-types included in the vaccine was not affected by the immunization. It was later found that the hyperimmune immunoglobulin persisted longer than expected in the serum, which may be of interest from a clinical point of view, but difficult to explain (McClain et al., 2001). A problem faced by anticapsular vaccination is the variability of capsular antigens in the natural *Klebsiella* populations. The selection of vaccine serotypes in the 24-valent vaccine was based on the most prevalent serotypes from bacteremic infections in Europe and the United States (Cryz et al., 1986). However, the serotypes included in this vaccine represent only 29% of the strains found in Taiwan (Fung et al., 2000) and do not include serotype K1, which is frequent in Asia. The polyvalent vaccine is likely to protect against *E. aerogenes* (Cryz et al., 1990) because of immunological crossreactions.

Vaccination against the *Klebsiella* O-antigen variation is attractive for several reasons. First, it is much less variable than the K-antigen, with only nine distinct serotypes (Fujita and Matsubara, 1984; Trautmann et al., 1996; Hansen et al., 1999) and with serotype O1 representing a large fraction of the isolates (e.g., nearly 40% of the isolates in the study of Trautmann et al., 1997). Second, in spite of the presence of the thick capsule, the O-antigens are exposed to the surface (Tomas et al., 1991), and anti-O1 monoclonal antibodies were shown to be protective in experimental models (Mandine et al., 1990; Rukavina et al., 1997).

Other candidate vaccines against *Klebsiella* infection or its pathogenic effects include a cytotoxin toxoid (Singh and Sharma, 2001), a conjugate made of O-polysaccharide and iron-regulated cell surface proteins (Chhibber and Bajaj, 1995), and hepta- or monovalent bacterial lysates (Kuenen et al., 1994).

The *Klebsiella* outer membrane protein A (KpOmpA) was shown to have properties of a carrier protein when conjugated to *Streptococcus pneumoniae* polysaccharides (Libon et al., 2002) and is suitable for nasal immunization (Goetsch et al., 2001). KpOmpA interacts with antigen-presenting cells by activating them, triggering cytokine production by macrophages and dendritic cells (Jeannin et al., 2002).

### Antibiotic Susceptibility

In the last decades, the levels of antibiotic resistance of *Klebsiella* strains involved in nosocomial infections has been changing rapidly for many antimicrobial classes and compounds, in particu-

lar for the successive generations of  $\beta$ -lactam antibiotics. For figures on the prevalence of antimicrobial resistance in different clinical settings and infection types, the reader is referred to recent publications (Livermore, 1995; Bauernfeind, 1996; Brisse et al., 2000; Paterson et al., 2000; Jones, 2001; Paterson, 2001; Stock and Wiedemann, 2001; Bouza and Cercenado, 2002; Gupta, 2002; Paterson, 2002; Paterson et al., 2003).

Strains of *Klebsiella* are naturally resistant to aminopenicillins (ampicillin and amoxicillin) and carboxypenicillins (carbenicillin and ticarcillin) and other penicillins but susceptible to most other  $\beta$ -lactam antibiotics. This natural resistance is due to the low-level constitutive production of a chromosomal class A  $\beta$ -lactamase, which is inhibited by clavulanic acid. Narrow-spectrum cephalosporins (cephalotin and cephalixin) can also be inactivated, although their minimum inhibitory concentrations (MICs) are low (Livermore, 1995). The chromosomal  $\beta$ -lactamase genes are known as *bla<sub>SHV</sub>* and *bla<sub>LEN</sub>* in *K. pneumoniae* (Arakawa et al., 1986; Hæggman et al., 1997; Chaves et al., 2001) and *bla<sub>OXY</sub>* in *K. oxytoca* (Arakawa et al., 1989; Fournier et al., 1996). In *K. pneumoniae*, *bla<sub>SHV</sub>* is found in strains of phylogenetic group KpI (Brisse and Verhoef, 2001), whereas *bla<sub>LEN</sub>* is the chromosomal gene of KpIII strains (Hæggman et al., 2004). KpII strains harbor a new variant of the gene named “*bla<sub>OKP</sub>*” (Hæggman et al., 2004). At least three groups of *bla<sub>OXY</sub>*  $\beta$ -lactamase genes can be distinguished (Fournier et al., 1996; Granier et al., 2003a; Granier et al., 2003b). In *K. oxytoca*, the chromosomal  $\beta$ -lactamase can be overexpressed, leading to a characteristic antibiogram showing a high resistance to all the penicillins except temocillin, resistance to cefuroxime, aztreonam, cefotaxime and ceftriaxone, but susceptibility (in contrast to isolates producing ESBL enzymes) to ceftazidime. Overexpression has been attributed to mutations in the promoter sequences of the *bla<sub>OXY</sub>*  $\beta$ -lactamase genes (Fournier et al., 1995). Because of the very high amounts of enzyme expressed, hyperproducers are often resistant to the presence of  $\beta$ -lactamase inhibitors (Livermore, 1995). Hyperexpression of chromosomal *bla<sub>OXY</sub>* genes has been found in 10–20% of *K. oxytoca* European clinical isolates (Liu et al., 1992; Reig et al., 1993; Livermore and Yuan, 1996).

In *K. pneumoniae*, higher levels and wider spectra of resistance to  $\beta$ -lactams mostly result from the production of plasmid-mediated  $\beta$ -lactamases, often SHV1 and its ESBL derivatives. The strains producing SHV1 remain susceptible to third generation cephalosporins (e.g., cefotaxime). Since 1983 (Knothe et al., 1983), the emergence of resistance of *K. pneumoniae* and

*K. oxytoca* isolates to third-generation cephalosporins (cefotaxime and ceftazidime) and aztreonam has been reported with increasing frequency (Kliebe et al., 1985; Buré et al., 1988; Jarlier et al., 1988; Gutmann et al., 1989; Podbielski et al., 1991; Paterson et al., 2003). Resistance is most often mediated by plasmids with various alterations of the SHV1, TEM1 and TEM2  $\beta$ -lactamases (Barthélémy et al., 1988). (The name “TEM” is a contraction of Temoniera, the name of a patient from whom the first resistant bacteria known to contain TEM  $\beta$ -lactamases were isolated.) More than 50 amino-acid sequence variants of SHV and more than 120 variants of TEM have been described and are compiled on the Internet on the Lahey Clinic Web site (<http://www.lahey.org>). At least 50 such variants have been described in *K. pneumoniae* (Bouza and Cercenado, 2002). The evolution of  $\beta$ -lactamase resistance and corresponding  $\beta$ -lactamases have been the subject of several excellent recent reviews (Livermore, 1995; Heritage et al., 1999; Tzouveleakis and Bonomo, 1999; Bradford, 2001; Helfand and Bonomo, 2003), to which we refer the interested readers.

ESBLs are most commonly found in *K. pneumoniae*. A surveillance study in the United States indicated increased MICs (2  $\mu$ g/ml or more) of ceftazidime for 24% of *K. pneumoniae* and 15% *E. coli* isolates (Jones et al., 1998). In a recent multicenter survey, phenotypic evidence for ESBL production was found in 73 (16%) out of 455 bloodstream isolates collected in the years 1996–1997 (Paterson et al., 2003), most isolates showing evidence for production of more than one  $\beta$ -lactamase (mean 2.7, range 1–5). The most common ESBL was SHV (in 49 of the 73 producers), whereas TEM was found in 12 ESBL producers, and CTX-M type ESBLs in 17 ESBL producers. These latter ESBL types, which are much more active against cefotaxime than against ceftazidime, pose a problem of detection, as screening procedures for ESBL production often rely on ceftazidime resistance.

New  $\beta$ -lactam resistance phenotypes of concern that have been described in *Klebsiella* include carbapenem-hydrolyzing enzymes (Bradford et al., 1997; Ahmad et al., 1999; Livermore and Woodford, 2000; Yigit et al., 2001), including one produced by a variant of the chromosomally-encoded *bla<sub>SHV1</sub>* gene (Poirel et al., 2003), and plasmid-mediated *ampC*  $\beta$ -lactamases, which are generally resistant to  $\beta$ -lactamase inhibitors (Bradford et al., 1997; Rupp and Fey, 2003). However, at present carbapenems are very active in vitro against the vast majority of *Klebsiella* strains.

*Klebsiella* isolates remain generally susceptible to quinolones, trimethoprim-sulfamethoxazole, and aminoglycosides (Watanabe et al., 1980;



Stock and Wiedemann, 2001; Bouza and Cercenado, 2002). However, plasmids mediating aminoglycoside-modifying enzymes have spread in the last three decades and are often carrying ESBL genes as well (Fernandez-Rodriguez et al., 1992; Paterson 2001; Sarno et al., 2002). An association between ESBL production and quinolone resistance has also been described (Brisse et al., 2000; Paterson et al., 2000). Quinolone resistance is generally determined by mutations in the chromosomal genes *gyrA* and *parC* (Deguchi et al., 1997; Brisse et al., 1999) but can also be encoded on plasmids (Martinez-Martinez et al., 1998).

### Genomics

The genome of one *K. pneumoniae* strain, MGH 78578, which belongs to *K. pneumoniae* phylogenetic group KpI, has been partially sequenced by the Washington University Genome Sequencing Center (McClelland et al., 2000). A whole genome shotgun approach was used to generate an approximately 8x coverage. The genome sequence is not annotated yet, but BLAST searches can be performed (at the <http://www.genome.wustl.edu/blast/client.pl> {Genome Sequencing Center of Washington University in St. Louis Web site}). Comparison of a 3.9x coverage of the *K. pneumoniae* genome with the sequenced *E. coli* K12 genome identified 2423 common genes with an average of 82% nucleotide similarity (McClelland et al., 2000). Genes common to *E. coli* K12 and *K. pneumoniae* but absent in the three *S. enterica* serovars Typhi, Typhimurium, and Paratyphi A, were found.

The genes common to *E. coli* K12 and the *K. pneumoniae* maize endophyte strain 342, which belongs to phylogenetic group KpIII (Dong et al., 2003), were investigated by hybridization on a microarray containing 96% of the annotated ORFs of *E. coli* K12 (Dong et al., 2001). Three thousand genes were found to be common between the two organisms, using a threshold of 55% nucleotide similarity, whereas 1030 *E. coli* genes were absent in strain 342. This approach, however, does not reveal the genes of *K. pneumoniae* that are not present in *E. coli*. The results were congruent with the known physiological characteristics of the two strains. For example, strain 342 lacks a homolog of the *E. coli* tryptophanase gene responsible for indole formation from tryptophan, and genes necessary for flagellum biosynthesis and function are missing in the *K. pneumoniae* strain 342 (Dong et al., 2001).

### Typing Methods

*Klebsiella* species are genetically heterogeneous and strains within species and subspecies can be discriminated by a number of methods including

biochemical tests, analysis of antigenic specificities, bacteriophage susceptibility typing, bacteriocin susceptibility or production typing, and molecular typing methods.

**BIOTYPING** Several tests, giving different reactions within a collection of *K. pneumoniae* strains, have been used empirically to biotype *K. pneumoniae* and other *Klebsiella* species. Although much less powerful than the other typing methods, biotyping can be performed in many laboratories and can provide ecologically meaningful information. However, nowadays, biotyping is not commonly used for typing.

The fermentation of dulcitol, D-tartrate, citrate and mucate, urea splitting, and the Voges-Proskauer test have been proposed for biotyping by Kauffmann (1949). Ørskov (1957) found 26 biotypes among 125 indole- and gelatin-negative strains belonging to 68 K-antigen types based on the dulcitol, adonitol, sorbose, urease, D-tartrate and sodium citrate tests; and 10 different biotypes among 50 indole- and gelatin-positive strains of 29 capsule types. Duncan and Razzell (1972) found 29 distinct biotypes using indole, Voges-Proskauer; lactose, sucrose and dulcitol fermentation; malonate, citrate, and D-gluconate utilization; lysine and ornithine decarboxylase; and urea hydrolysis tests in agar media inoculated with a modified Steers replicator. Barr (1978) observed large variation in the metabolism of most substrates examined, and his biotyping system used different concentrations of carbohydrate substrates, and citrate, malonate and urea tests. A biotyping system has been used by C. Richard at the Institut Pasteur (Richard, 1973; Richard, 1982). The tests included sorbose, dulcitol, L-tartrate fermentation, and secondarily rhamnose and adonitol fermentation. For biotype determination in *K. pneumoniae* subsp. *pneumoniae*, mucate, malonate utilization, and urea hydrolysis, or growth at 44°C were found useful (Richard, 1985). For typing *K. oxytoca*, D-melezitose fermentation, tetrathionate reductase activity, brown pigment production on a mineral gluconate-ferric citrate medium were found useful (Richard and Monteil, 1987). A biotyping method based on carbon source utilization tests is under development for *K. pneumoniae*. Among the selected tests, utilization of 5-ketogluconate could discriminate between plant-associated strains (utilization positive) and clinical strains (no utilization; P. A. D. Grimont and E. Ageron, unpublished observation). Finally, a method based on the measurement of the kinetics of 32 metabolic reactions performed in microtiter plates has been developed and is commercially available (Tullus et al., 1999).

Biotyping has a potential value in epidemiological studies when used in conjunction with

capsular serotyping. Use of the two methods together allows the recognition of many more types than is possible with only one of the methods (Rennie and Duncan, 1974).

**SEROTYPING** Serotyping of *Klebsiella* strains, which are nonmotile, is based on the characterization of the O- and K-antigens.

O-serotyping has long been technically difficult to perform because the presence of heat-stable K-antigens makes determination of O-antigens difficult (Ørskov and Ørskov, 1984b). One way around this problem is to generate non-capsulated variants by subculturing in a nutrient broth containing 50% bile. Because only nine O-antigens have been described (Fujita and Matsubara, 1984; Trautmann et al., 1996; Hansen et al., 1999), O-typing is considered less useful than K-typing for molecular epidemiology and outbreak investigation. However, the increasing levels of antimicrobial resistance in *Klebsiella* strains have renewed interest in O-antigens as potential vaccines (see the section Human Vaccine in this Volume). In addition, O-typing can subdivide K-types, and the development of a modified enzyme-linked immunosorbent assay (ELISA) for O-antigen determination (Alberti et al., 1993; Trautmann et al., 1996) has rendered O-typing easier to perform.

Capsule serotyping or K-typing is presently one of the preferred methods for typing *Klebsiella* strains, as it is highly discriminatory and the only definitive typing method available (Hansen et al., 2002). Unfortunately, this technique is only available in reference centers. An alternative to serotyping that targets the genetic region involved in capsular polysaccharide synthesis was developed recently (Brisse et al., 2004a).

Following the pioneering work of Julianelle (1926), who described the first two capsular serotypes, a total of 82 K-antigens (K1–K82) were described. After deletion of five antigens (K73 and K75–K78), 77 K-antigens are now included in the international K-typing scheme (Ørskov and Fife-Asbury, 1977; Ørskov and Ørskov, 1984b). Antisera used for *Klebsiella* capsular typing can also type *E. aerogenes* (Richard, 1977).

The majority of K-antigens contain only a charged monosaccharide constituent, most often glucuronic acid and hexoses. However, non-carbohydrate constituents (such as formyl or acetyl group and ketal-linked pyruvate) are found: these may function as antigenic determinants and be the cause of crossreactions of some polysaccharides (Edmonston and Cooke, 1979b; Ørskov and Ørskov, 1984b; Ewing, 1986). Crossreactions between K-antigens are numerous and it is necessary in several cases to absorb the antisera for diagnostic use.

Several typing procedures may be used: 1) The capsular swelling reaction or Quellung reaction (Kauffmann, 1949; Edwards and Ewing, 1972; Richard, 1973; Ørskov and Ørskov, 1984b) is the most often used. Cultures on carbohydrate-rich media (e.g., Worfel-Ferguson medium) yield thicker capsules. The test is performed by mixing equal volumes of the bacterial suspension ( $1 \times 10^8$  colony-forming units per ml) and the antiserum (pooled-, specific-, or absorbed antiserum) on a glass slide. After application of a coverslip, the preparation is examined under the ordinary light microscope or the phase-contrast microscope using an oil immersion objective. The capsule-antibody precipitate becomes refractile and visible under the microscope. 2) In the indirect immunofluorescence test, bacteria are incubated with a capsular antiserum (from rabbit), then incubated with a sheep anti-rabbit fluorescent conjugate, and examined under a fluorescence microscope (Riser et al., 1976a; Riser et al., 1976b). 3) A double diffusion gel precipitation (Ouchterlony) test used with carefully absorbed sera has been found to give straightforward results (Mori et al., 1989). 4) A countercurrent immunoelectrophoresis technique was reported to be more specific, more economical, and less time-consuming than the capsular swelling method (Palfreyman, 1978). It is currently in use at the World Health Organization Collaborative Center on *E. coli* and *Klebsiella* (Hansen et al., 2002). 5) The antibody-coated *Staphylococcus* coagglutination test (Onokodi and Wauters, 1981) uses *Staphylococcus* Cowan I, which contains large amounts of protein A on its surface. Protein A binds antisera, and the suspension of staphylococcal cells coated with specific antisera becomes a reagent for specific coagglutination with corresponding *Klebsiella*. And finally, 6) the latex agglutination test is an alternative method in which latex particles are used in place of *Staphylococcus* Cowan I (Onokodi and Wauters, 1981).

Crossreactions have been noted between *Klebsiella* K-antigens and *E. coli* K-antigens (Kauffmann, 1949) because of (in some cases at least) the horizontal transfer of the genomic region responsible for capsule synthesis (see the section Properties Relevant to Pathogenicity for Humans in this Chapter).

**MOLECULAR SEROTYPING** In an effort to replace classical K-serotyping, restriction of the amplified capsular antigen gene cluster was recently developed (Brisse et al., 2004a). The method enables to determine the capsular antigen without using antiserum. PCR amplification of the *cps* genomic region is followed by restriction with enzyme *HincII* and agarose gel electrophoresis, generating C-patterns. The C-patterns

obtained for strains of any K-serotype were different from those of all other K-serotypes, with only one exception (K22 and K37). More than 100 C-patterns were distinguished; in general, strains of the same K-type but belonging to distinct *Klebsiella* species showed distinct C-patterns. Only 4.5% of the strains were non-typable owing to PCR amplification failure. Non-capsulated strains were amenable to molecular K-serotyping (Brisse et al., 2004a).

**PHAGE TYPING** Bacteriophages have been isolated that depolymerize capsular material (Ørskov, 1984a). Different phage typing systems have been proposed (Slopek et al., 1967b; Slopek, 1978; Rennie et al., 1978; Gaston et al., 1987). These systems could type 70–96% of *Klebsiella* isolates. Reproducibility was often good when replicate testing was done immediately, and poor when replicate testing was done after a few weeks (Gaston et al., 1987). Phage typing has been compared to K-typing and re-evaluated recently (Sechter et al., 2000). The discriminatory power of phage typing was comparable to that of K-typing, but phage types were found to be less stable and reproducible. Phage typing is not used frequently; the last *Klebsiella* outbreak investigation making use of this approach was published in 1996 (French et al., 1996).

**BACTERIOCIN TYPING** Bacteriocins are the most abundant and diverse of the microbial defense systems (Riley and Wertz, 2002). Bacteriocins of *Klebsiella* were first characterized in detail by Hamon and Péron (1963). Bacteriocins of *Klebsiella* have been called “pneumocins” (Hamon and Péron, 1963), “klebocins” (Buffenmeyer et al., 1976; Walia et al., 1988), “klebecins” (Edmonston and Cooke, 1979a) or simply “bacteriocins of *Klebsiella*” (Hall, 1971). Bacterial isolates can be typed either by the sensitivity to bacteriocins produced by a set of strains (bacteriocinotyping) or by the pattern of growth inhibitor exerted by their bacteriocins on a set of indicator strains (bacteriocinogenotyping). About 34–51% of isolates produce bacteriocins either spontaneously or after induction by mitomycin (Hamon and Péron, 1963; Bauernfeind et al., 1981). Different bacteriocin typing systems were proposed that could type 59–97% of isolates (Slopek and Maresz-Babczyszyn, 1967a; Buffenmeyer et al., 1976; Edmonston and Cooke, 1979a; Israil, 1980b; Bauernfeind et al., 1981). Reproducibility of these systems can be poor. Reproducibility can be improved by a careful evaluation of each technique (Bauernfeind, 1984). *Klebsiella* strains have also been typed by production of and sensitivity to colicins as determined by the use of standard colicin indicator strains and colicin producing strains (Israil,

1980a). One plasmid encoding klebicin B of *K. pneumoniae* was sequenced, and it was found that klebicin B has sequence similarity to DNase pyocins and colicins, suggesting that this klebicin is a nonspecific endonuclease (Riley et al., 2001).

**MULTILOCUS ENZYME ELECTROPHORESIS** Multilocus enzyme electrophoresis (MLEE) consists of monitoring the variation, among strains, of the electrophoretic migration of amino acid sequence variants of a number of enzymes involved in housekeeping functions (Selander et al., 1986). In striking contrast to the situation in *E. coli* and *Salmonella*, MLEE has almost never been used for *Klebsiella* typing and population genetics, although occurrence of variation was early demonstrated among *Klebsiella* strains (Goulet, 1980). An alternative method to index MLEE variation, which involves polyacrylamide-agarose gel electrophoresis followed by electrophoretic transfers on nitrocellulose sheets, was developed (Nouvellon et al., 1994; Combe et al., 2000). To our knowledge, the method was applied only to the investigation of one outbreak of one ESBL-producing *Klebsiella* strain (Nouvellon et al., 1994). Because of the paucity of MLEE studies of *Klebsiella* species, basic aspects of their population genetics, such as the amount of genetic diversity and the evolutionary impact of genetic recombination within species, are still ignored at the present time.

**MOLECULAR TYPING METHODS** Molecular typing methods aim at discriminating among strains based on the differences in their genomic DNA sequence and organization (for a recent review, see Van Belkum et al., 2001). Over the last years many molecular typing methods have been applied to investigate outbreaks due to *Klebsiella* strains and to compare strains from different time points and geographic origins. Most methods rely on DNA fragment fingerprints, which can be highly discriminatory but are generally not standardized enough to allow comparison of data from distinct studies. Sequence-based methods such as multilocus sequence typing (MLST; Maiden et al., 1998), which have the advantage of standardization and portability, are only beginning to be applied to *Klebsiella*. Six genes (*tonB*, *ureD*, *tyrB*, *aroA*, *gapA* and *rpoB*) that could be included in an MLST typing scheme and have been sequenced in a number of *K. pneumoniae* strains, are available in GenBank (Z. Y. Shi, unpublished observation). A full MLST scheme based on genes *aroA*, *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB* was just developed (S. Brisse, unpublished), and data are available on the public MLST Web site of Mark Achtman's group at (<http://web.mpiib-berlin.mpg.de/mlst/>).

**PLASMID PROFILING.** Plasmid DNA profiles and plasmid DNA restriction profiles have been seldom used in epidemiological investigations (Walia et al., 1988; Marranzano et al., 1996). Compared to other methods, however, plasmid profiling is particularly useful to track the spread of plasmids encoding antimicrobial resistance genes among strains and species, as opposed to strain epidemics (Bingen et al., 1993).

**RANDOM PCR METHODS.** Methods based on the PCR amplification of uncharacterized DNA fragments, such as random amplified polymorphic DNA (RAPD), repeat-based PCR (rep-PCR) or enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR), present the advantage of being applicable without prior knowledge of the genome sequence and with few technical tuning steps. These methods can, therefore, be very helpful for rapid local investigations of the sources and routes of clonal spread. Applications to *Klebsiella* epidemiology have been published (Wong et al., 1994; Eisen et al., 1995; Gori et al., 1996; Lhopital et al., 1997; Shannon et al., 1998; Ben-Hamouda et al., 2003). Efforts to standardize RAPD have been made (Vogel et al., 1999). However, these methods generally suffer from poor reproducibility, especially when interlaboratory comparisons are concerned (Van Belkum et al., 2001). Multiple-primer RAPD has proved very useful as a screening method to identify divergent lineages within *K. pneumoniae* and *K. oxytoca* (Brisse and Verhoef, 2001).

**PULSE-FIELD GEL ELECTROPHORESIS.** Pulse-field gel electrophoresis (PFGE), or DNA macrorestriction fragment analysis, is probably the most widely used DNA fingerprinting method in bacterial typing, and this holds true for *Klebsiella* (Poh et al., 1993; Arlet et al., 1994; Gouby et al., 1994; Gori et al., 1996; Monnet et al., 1997; Toldos et al., 1997; Yuan et al., 1998; Chang et al., 2000; Nadjar et al., 2000; Sechi et al., 2001; Cheng et al., 2002; Hansen et al., 2002; Lebessi et al., 2002). It consists of cutting the genome with a restriction enzyme, followed by separation of the large fragments on an agarose gel by alternately applying two electric fields that are differently orientated. The restriction enzymes *HaeIII*, *XbaI* and *SpeI* have been used in *Klebsiella*, mostly with the purpose of investigating whether clinical isolates recovered during outbreaks of ESBL-producing *klebsiellae* belonged to a single clone (see above references). PFGE data have been compared to O:K serotyping data and shown to be more discriminatory (Hansen et al., 2002). These authors remind us that serotyping cannot be replaced by PFGE, though, given the low standardization and inter-laboratory reproducibility of this latter method.

**RIBOTYPING.** Ribotyping, or ribosomal ribonucleic acid (rRNA) gene restriction patterns (Grimont and Grimont, 1986; Stull et al., 1988; Bingen et al., 1994; Brisse, 2003), has been used extensively to type *Klebsiella* isolates (Bingen et al., 1993; Maslow et al., 1993; Thompson et al., 1993; Arlet et al., 1994; Lhopital et al., 1997; Sader et al., 1998; Brisse et al., 2000; Sechi et al., 2001; Cheng et al., 2002; Fung et al., 2002; Yu et al., 2002). It shows excellent discriminatory power in *Klebsiella* owing to the presence of several ribosomal RNA operons in the genome. The availability of the automated method making use of the riboprinter instrument (DuPont-Qualicon, Wilmington, DE) has rendered ribotyping extremely easy to perform and, above all, makes it one of the few standardized typing methods. Indeed, ribotyping data are highly reproducible from riboprinter to riboprinter and easily portable among laboratories (Brisse et al., 2002; Brisse, 2003). Enzyme *EcoRI* is most generally used for typing purposes. One particularly useful application of ribotyping is the identification of *Klebsiella* species and phylogenetic groups (Brisse and Verhoef, 2001).

**AFLP.** Often taken as an abbreviation for Amplification of Fragment Length Polymorphisms, but not initially named with this in mind, AFLP was first developed as a genetic tool for plant breeders (Vos et al., 1995) and is patented by the Dutch company KeyGene N.V. (Wageningen, The Netherlands). It is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. Its technical details and applications to microbial typing were reviewed by Savelkoul et al. (1999). Advantages of AFLP include very high discriminatory power, high reproducibility, flexibility in the sequence coverage of the genome, and the almost exclusive possibility to reamplify and sequence AFLP fragments of interest, thus facilitating the detection of DNA sequences associated with particular phenotypic traits (Van Belkum, 2003). AFLP recently has been evaluated and validated for the delineation of sets of strains implicated in clonal spread during *Klebsiella* hospital outbreaks and for the identification of *Klebsiella* species and phylogenetic groups (Jonas et al., 2004). A variation of the protocol that permits separation of AFLP fragments on agarose gels, rather than polyacrylamide gels as performed in the original method, has been developed for *Klebsiella* (Van der Zee et al., 2003).

**OTHER TYPING METHODS** Typing methods based on mass spectrometry are being increasingly evaluated for microbial identification, typing and proteomics (Shah et al., 2002). Only one application to *Klebsiella* was published (Jackson et al.,

1997). These methods, in particular matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), can be extremely cheap and quick compared to molecular typing methods and present the possibility to identify protein components associated with phenotypic aspects of the bacteria. However, interpretation of the fingerprints for typing purposes may remain a problem, as they may not be independent of the culture media used.

### Klebsiella Components Used as Biological Models

**NITROGEN FIXATION AND INTERACTION WITH PLANTS** Associative nitrogen fixation is carried out by bacteria living on the roots of nonleguminous plants. *Klebsiella pneumoniae*, *K. oxytoca* or *K. planticola* are capable of fixing nitrogen and are classified as associative nitrogen fixers, or diazotrophs (Mahl et al., 1965; Ladha et al., 1983). Endophytic *K. pneumoniae* strains found within maize are diazotrophic (Palus et al., 1996), and one studied strain was able to produce dinitrogenase reductase protein within roots, as long as a carbon source was added to the maize seedlings (Chelius and Triplett, 2000). These endophytic strains belong to *K. pneumoniae* phylogenetic group KpIII (Brisse and Verhoef, 2001) as shown by Dong et al. (2003).

Nitrogen fixation is catalyzed by the enzyme nitrogenase in the absence of combined nitrogen and under anaerobic conditions (Postgate, 1982; Schmitz et al., 2002). Nitrogen fixation is catalyzed by the enzyme complex nitrogenase and requires high amounts of energy in the form of two ATP molecules for each electron transferred to the catalytic site (Schmitz et al., 2002). In the facultative anaerobe *K. pneumoniae*, nitrogen fixation occurs only in anaerobic or microaerobic conditions. Low O<sub>2</sub> concentrations enhance nitrogenase synthesis; higher O<sub>2</sub> concentrations inhibit synthesis and irreversibly inactivate the enzyme complex (Hill et al., 1984). Molybdenum is required for the activity of nitrogenase (Shah and Brill, 1977; Shah et al., 1984), and it is found in this enzyme as part of low-molecular-weight cofactors (Johnson, 1980). Two different molybdenum-containing cofactors have been described: iron-molybdenum cofactor (FeMo-co) contained in component I (Shah and Brill, 1977), and molybdenum cofactor (Mo-co; Johnson, 1980). FeMo-co can activate in vitro nitrogenase from certain *nif* mutants (Roberts et al., 1978) and is believed to be an active site of nitrogen reduction (Shah et al., 1978). Nitrogenase is composed of two different proteins—nitrogenase component I and nitrogenase component II. The two subunits of component I are coded by the genes *nifK* and *nifD*, which share

an operon with the component II gene (*nifH*). There are at least 17 contiguous nitrogen fixation (*nif*) genes clustered on the chromosome and organized into eight operons, whose products are likely to be required for the synthesis and assembly of an active nitrogen-fixing system (Roberts and Brill, 1981; Beynon et al., 1983; Ausebel, 1984). These genes include *nifHDK* (nitrogenase structural components); *nifF* and *nifJ* (electron transport components); *nifQ*, *nifB*, *nifE*, *nifN* and *nifV* (FeMo-co biosynthetic components); *nifA* and *nifL* (positive and negative regulatory elements); *nifM* (Fe protein maturation component); and *nifY*, *nifX*, *nifU* and *nifS* (functions not known).

The genetic control of nitrogen fixation is complex (Roberts and Brill, 1981; Gussin et al., 1986) and has been reviewed recently (Schmitz et al., 2002). The products of the *nifLA* operon regulate the transcription of the other operons. NifA is a transcriptional regulator that acts by promoting the formation of transcriptionally-productive open complexes between  $\sigma$ -54 and the polymerase holoenzyme (Morett and Buck, 1989; Hoover et al., 1990). NifL modulates the activity of NifA by direct protein-protein interaction in response to the presence of combined nitrogen and molecular oxygen (Henderson et al., 1989; Govantes et al., 1998). The primary oxygen sensor appears to be the global regulator Fnr (fumarate nitrate reduction regulator), which transduces the signal to NifL (Grabbe et al., 2001). The nitrogen signal is mediated via the GlnK protein, which relieves NifL inhibition under nitrogen-limiting conditions (He et al., 1998; Jack et al., 1999). *Klebsiella pneumoniae* apparently senses external nitrogen limitation as a drop in the internal glutamine pool (Schmitz, 2000).

**UREASE** Urease from *K. pneumoniae* plays an important role in nitrogen metabolism and is intensely studied as a model to understand urease catalytic activity. This nickel-containing enzyme hydrolyzes urea to ammonia and carbon dioxide. Its regulation has been characterized (Friedrich and Magasanik, 1977; Magasanik, 1982), and the three-subunit enzyme shown to contain four nickel ions per native molecule (Todd and Hausinger, 1987). Urease was localized to the cytoplasmic portion of the cell (Mulrooney et al., 1989). The crystal structure of *K. pneumoniae* urease has been the first to be determined, and revealed a unique dinuclear active site with the metal ions bridged by a carbamylated lysine residue (Jabri et al., 1995; Pearson et al., 1997). Proper assembly of this metallocenter is a key step for maturation of urease and, in *K. pneumoniae*, involves the products of the *ureD*, *ureE*, *ureF* and *ureG* accessory genes located

adjacent to the structural genes (*ureA*, *ureB* and *ureC*; Soriano and Hausinger, 1999).

**SECRETION** Pullulanase (pullulan 6-glucanohydrolase), an extracellular starch-debranching enzyme produced by some strains of *K. pneumoniae*, hydrolyzes the (1-6)- $\alpha$ -glucosidic linkages in pullulan and starch to form maltotriose (Wöhner and Wöhner, 1978). Pullulanase has been intensely analyzed as a model protein substrate for the general secretory pathway of Gram-negative bacteria (Pugsley et al., 1997). Pullulanase is first exported to the cell surface where it accumulates until it is released into the growth medium. Production is induced by the substrate of the enzyme (pullulan), or a product of the enzyme (maltose or maltodextrin) but is repressed in the presence of glucose. The structural gene for pullulanase was studied by Michaelis et al. (1985). The nucleotide sequence of the pullulanase gene, required for pullulanase secretion, was determined by Katsuragi et al. (1987) for *pulA* and by Pugsley and Reyss (1990) for *pulL*, *pulM*, *pulN* and *pulO*. Another starch utilization pathway was discovered more recently in *K. oxytoca* (Fiedler et al., 1996). It involves the extracellular conversion of starch to cyclodextrins, uptake of these, and intracellular linearization by a cyclodextrinase.

**PECTIN DEGRADATION** *Klebsiella oxytoca* isolates (previously, indole-positive *K. pneumoniae*), without known phytopathogenic relationship to plants, are able to digest the kind of calcium-stabilized polygalacturonate gels used in studies of soft rot *Erwinia* species (Von Riesen, 1976). These bacteria showed detectable, albeit generally weak, ability to digest polygalacturonic (pectic) acid (Starr et al., 1977). Analyses of the pectolytic enzyme contents of the cells and culture supernatants of *K. oxytoca* revealed that readily detectable quantities of cell-bound polygalacturonic acid *trans*-eliminase and hydrolytic polygalacturonase were found but not excreted rapidly and massively into the growth medium, unlike soft-rot *Erwinia* species. The *pehX* gene, encoding the enzyme polygalacturonase that cleaves a polygalacturonic chain of demethoxylated pectin, was cloned (Kovtunovych et al., 2000) and used as a basis for a *K. oxytoca*-specific PCR assay (Kovtunovych et al., 2003).

**HISTIDINE DECARBOXYLASE** Inducible histidine decarboxylase (HDC) from *K. planticola* is responsible for histamine formation from histidine in food products, which can result in a syndrome called "histamine fish poisoning" (Guirard and Snell, 1987). HDC requires pyridoxal 5'-phosphate as a coenzyme and decarboxylates

only L-histidine among the amino acids derived from protein (Guirard and Snell, 1987). The genes encoding pyridoxal phosphate-dependent histidine decarboxylases from *K. planticola* and *E. aerogenes* have been cloned (Kamath et al., 1991). Although histamine fish poisoning was previously attributed to *K. pneumoniae* and *K. oxytoca*, Kanki et al. showed that this conclusion was due to misidentification of *K. planticola* and *K. ornithinolytica* (Kanki et al., 2002).

## Klebsiella and Biotechnology

**BIOCONVERSIONS** *Klebsiella pneumoniae* has the ability to grow fermentatively on glycerol without an exogenous hydrogen acceptor (Lin, 1976). This is achieved by the cooperation of two pathways specified by the *dha* regulon (Lin, 1976). In the oxidative pathway, glycerol is dehydrogenated by the nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-linked glycerol dehydrogenase to dihydroxyacetone, which is then phosphorylated by the dihydroxyacetone kinase to dihydroxyacetone phosphate. In the reductive pathway, glycerol dehydratase produces 3-hydroxypropionaldehyde, which is converted to 1,3-propanediol (lost to the growth medium) by a NADH-linked oxidoreductase (Lin, 1976). In the presence of semicarbazide and with adjusted aerobic and substrate conditions, 3-hydroxypropionaldehyde can be accumulated from glycerol by *K. pneumoniae* (Slininger and Bothast, 1985). This bioconversion is interesting since the chemical oxidation of 3-hydroxypropionaldehyde leads to acrylic acid, an industrially important polymerizable monomer currently derived from petroleum (Slininger and Bothast, 1985).

Production of 1,3-propanediol by *Klebsiella* (Homann et al., 1990) has attracted renewed interest because of the industrial need to find precursor monomers for the synthesis of a new polyester used in carpets and textiles, polytrimethylene terephthalate (Huang et al., 2002). 1,3-Propanediol is the characteristic fermentation product of glycerol and is formed together with acetic acid in a ratio of 2 to 1. *Klebsiella pneumoniae* can shift from acetic acid to 2,3-butanediol formation under conditions of decreased pH (Biebl et al., 1998). However, the cost of glycerol being elevated, cost-effective production of 1,3-propanediol can only be achieved if a cheaper substrate, such as glucose, is used. Efforts are being made to combine the pathway of glucose to glycerol with that from glycerol to 1,3-propanediol, either by combining microorganisms in simultaneous or two-step cultures or by creating recombinant microorganisms with both pathways (Biebl et al., 1999; Huang et al., 2002).

The production of 2,3-butanediol from lignocellulosic wastes is an alternative approach in the

conversion of biomass substrates to liquid fuels and chemical feedstocks (Johansen et al., 1975; Magee and Kosaric, 1987). To be economically attractive, the efficient utilization of all the available substrates in the cellulose and hemicellulose component of the biomass must be achieved (Yu and Saddler, 1983). Hemicellulose makes up 30–40% of agricultural residues. *Klebsiella pneumoniae* or *K. oxytoca* have been shown to utilize all of the major sugars (hexoses, pentoses, and certain disaccharides) and uronic acids derived from the hydrolysates of hemicellulosic and cellulosic materials (Yu and Saddler, 1983). When *K. oxytoca* (referred to as *K. pneumoniae*) was grown under finite air conditions in the presence of acetic acid, 50 g of D-glucose and D-xylose per liter could be converted to 25 and 27 g of butanediol per liter, respectively (Yu and Saddler, 1983). A maximum of 84.2 g of butanediol per liter was obtained with an initial glucose concentration of 262.6 g per liter (Qureshi and Cheryan, 1989). By a sequential coculture approach involving a cellulolytic and xylanolytic fungus, *Trichoderma harzianum* and *K. oxytoca* (referred to as *K. pneumoniae*), cellulose and hemicellulose substrates (or steam-exploded wood) were converted to 2,3-butanediol (Yu et al., 1985). Alternatively, *K. planticola* can produce large amounts of ethanol instead of butanediol after genetic recombination involving a multicopy plasmid carrying the pyruvate decarboxylase gene from *Zymomonas mobilis* (Tolan and Finn, 1987). The production of butanediol and ethanol is seen as a way to utilize pyruvate that avoids the production of acidic end products. Production of butanediol is enhanced in the presence of acetate, when oxygen is limited and the pH lowered. The pathway that utilizes two moles of pyruvate to produce one mole of butanediol involves three enzymes:  $\alpha$ -acetolactate synthetase,  $\alpha$ -acetolactate decarboxylase, and acetoin reductase (Magee and Kosaric, 1987). The genes encoding these enzymes are organized in an operon (*budABC*) and have been cloned and sequenced in *K. terrigena* (Blomqvist et al., 1993) and *K. pneumoniae* (Wardwell et al., 2001). A *Clostridium acetobutylicum* strain, into which the acetoin reductase gene from *K. pneumoniae* was transferred, produced butanediol only if acetoin was added to the culture medium (Wardwell et al., 2001). The *budABC* operon is under the control of BudR, a transcriptional regulator belonging to the LysR family, which was cloned and sequenced in *K. terrigena* (Mayer et al., 1995). The gene *budR* is separated from the *budABC* operon by a 106-bp intergenic region and is inversely oriented. BudR activity is induced by acetate and repressed by the global regulator Fnr (fumarate nitrate reduction regulator; Mayer et al., 1995).

The crystal structure of *K. pneumoniae* acetolactate synthase was determined (Pang et al., 2004).

**BIOREMEDIATION** Many environmental *Klebsiella* strains have been found with various metabolic properties that are interesting for bioremediation. Two early examples include herbicide and pesticide degradation. First, a soil isolate identified as *K. pneumoniae* subsp. *ozaenae* (but possibly rather a *K. pneumoniae* subsp. *pneumoniae* with low metabolic activity), which was found to convert 0.05% bromoxynil (3, 5-dibromo-4-hydrobenzotrile), a widely used herbicide, to 3, 5-dibromo-4-hydrobenzoic acid, and utilize the liberated ammonia as sole nitrogen source (McBride et al., 1986). This transformation is due to a plasmid-encoded nitrilase highly specific for bromoxynil (Stalker et al., 1987). A gene, designated “*bxn*,” encoding the bromoxynil-specific nitrilase, was cloned (Stalker et al., 1987). Second, *K. pneumoniae* was found to be able to reduce the organophosphorus pesticide fensulfothion to fensulfothion sulfide and 4-methylsulfanylphenol to 4-methylthiophenol, under oxygen limitation (Mac Rae and Cameron, 1985). More recently, *Klebsiella* strains were isolated with potential for bioremediation of phenol (Heesche-Wagner et al., 1999), endosulfan (Kwon et al., 2002), mercury (Essa et al., 2002), nitroglycerin (Marshall and White, 2001), cadmium (Sharma et al., 2000), aliphatic and aromatic nitriles (Nawaz et al., 1992), and acrylonitrile (Nawaz et al., 1991).

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## The Genus *Enterobacter*

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### Introduction

The genus *Enterobacter* was first proposed by Hormaeche and Edwards (1960a). However, the history of some species now placed in the genus *Enterobacter* can be traced, albeit with some confusion, to the end of the 19th century. “*Bacillus lactis aerogenes*” was isolated by Escherich (1885) from milk and renamed “*Bacillus aerogenes*” by Kruse (1896) and “*Aerobacter aerogenes*” by Beijerinck (1900). Until 1955, differentiation of this organism from Friedländer’s bacillus (now called *Klebsiella pneumoniae*) was not clear, and most authors considered “*B. lactis aerogenes*” or “*Aerobacter aerogenes*” to be either nonmotile or to contain both motile and nonmotile strains (Grimbert and Legros, 1900; Edwards and Fife, 1955). This led Edwards and Fife (1955) to state that “*A. aerogenes*” strains were in fact *Klebsiella* strains.

“*Bacterium cloacae*” was described by Jordan (1890) and transferred to a new genus “*Cloaca*” as “*Cloaca cloacae*” by Castellani and Chalmers (1920). In the first edition of *Bergey’s Manual* (Bergey et al., 1923), this species was transferred to the genus “*Aerobacter*” as “*A. cloacae*.” Since “*Aerobacter aerogenes*” was at that time indistinguishable from *Klebsiella pneumoniae*, Edwards and Fife (1955) proposed that the species “*A. aerogenes*” not be used, although disappearance of the type species (“*A. aerogenes*”) implied disappearance of the genus (“*Aerobacter*”). Because of this, two solutions were suggested for “*A. cloacae*”: 1) the reintroduction of the genus “*Cloaca*” with the species “*Cloaca cloacae*” (reference is not an exact match Kauffman, 1954); and 2) the redefinition of the genus “*Aerobacter*” with “*A. cloacae*” as type species (Edwards and Fife, 1955). However, this latter proposal did not conform to the rules of nomenclature.

A significant step forward occurred when Møller (1955) devised some simple methods for testing amino acid decarboxylases. Since the “*Cloaca*” group was arginine-positive it could

now easily be distinguished from the *Klebsiella* group, which was arginine-negative. This led to the finding of motile strains of the “*Cloaca*” group, which were arginine-negative and produced gas from inositol and glycerol (Hormaeche and Munilla, 1957). These strains were called “*Cloaca B*” (arginine-positive strains forming the “*Cloaca A* group”). Then, after reexamination of many cultures using the decarboxylase test, Hormaeche and Edwards (1958) redefined the genus “*Aerobacter*” to include two species, “*A. aerogenes*” (“*Cloaca B*”) and “*A. cloacae*” (“*Cloaca A*”), with the type species reaffirmed as “*A. aerogenes*.”

Grimes and Hennerty (1931) described a group of strongly proteolytic strains they named “*Aerobacter liquefaciens*.” However, this name was illegitimate since the same name had been used by Beijerinck (1900) for bacteria which could have been aeromonads, so that Grimes (1961) renamed his group as “*Aerobacter lipolyticus*.” Eventually, however, Beijerinck’s “*A. liquefaciens*” was placed on the list of rejected names (Judicial Commission, 1973), and “*A. liquefaciens*” Grimes and Hennerty became accepted.

In an attempt to avoid confusion resulting from the reclassification in the genus *Klebsiella* of many nonmotile strains previously labeled “*A. aerogenes*,” Hormaeche and Edwards (1960a 1960b) proposed a new genus *Enterobacter* as a substitute for “*Aerobacter*.” This genus was then composed of *E. cloacae* (the type species) and *E. aerogenes*. In 1963, the Judicial Commission of the International Committee on Nomenclature of Bacteria placed the name *Enterobacter* on the list of conserved names (Judicial Commission, 1963).

“*Aerobacter liquefaciens*,” included in the genus *Enterobacter* as *E. liquefaciens* (reference is not an exact match Edwards and Ewing, 1972), was then transferred to the genus *Serratia* as *S. liquefaciens* (Bascomb et al., 1971). DNA-relatedness studies have clearly demonstrated it to be a member of the genus *Serratia* (Steigerwalt et al., 1976).

A group of strains named *Hafnia* by Møller (1954) was included in the genus *Enterobacter* *E. hafniae* (Ewing and Fife, 1969) although it was

given in the 8th edition of *Bergey's Manual* (Buchanan and Gibbons, 1974) as *Hafnia alvei*. DNA-relatedness studies (Steigerwalt et al., 1976) clearly required the separation of *Hafnia alvei* from the genus *Enterobacter*.

The genus *Erwinia* has long been a depository for plant-associated members of the Enterobacteriaceae (see Chapter 154). Several species of this genus were found to be phenotypically similar to *Enterobacter* species (*Erwinia herbicola*, *E. ananas*, *E. uredovora*, *E. milletiae*, *E. dissolvens*, *E. nimipressuralis*) (reference is not an exact match Lelliott, 1974; reference is not an exact match Lelliott and Dickey, 1984) or highly related to *Enterobacter* species by DNA hybridization (*Erwinia dissolvens*, *E. nimipressuralis*) (Steigerwalt et al., 1976).

The other species now classified in the genus *Enterobacter* have been described after delineation by DNA-DNA hybridization. The species (and their synonyms) presently composing the genus *Enterobacter* are:

1. *Enterobacter cloacae* (Jordan) Hormaeche and Edwards 1960a; "*Bacillus cloacae*" Jordan 1890; "*Aerobacter cloacae*" (Jordan) Hormaeche and Edwards 1958. The type strain is strain ATCC 13047 (= CDC 279-56, NCTC 442.68, CIP 60.85). This strain is very close by DNA hybridization to the type strain of *E. dissolvens*.
2. *Enterobacter aerogenes* (Kruse) Hormaeche and Edwards 1960a; "*Bacillus aerogenes*" Kruse 1896; "*Aerobacter aerogenes*" (Kruse) Hormaeche and Edwards 1958; *Enterobacter aerogenes* (Hormaeche and Edwards 1960a 1960b); *Klebsiella mobilis* Bascomb et al. 1971. The type strain is strain ATCC 13048 (= CDC 819-56, NCTC 10006, CIP 60.86).
3. *Enterobacter gergoviae* Brenner et al. 1980; *Enterobacter* sp. urease positive (Richard et al., 1976). The type strain is strain ATCC 33028 (= CDC 604-77, CIP 76.01).
4. *Enterobacter sakazakii* Farmer et al. 1980; yellow-pigmented *Enterobacter cloacae* (Steigerwalt et al., 1976). The type strain is strain ATCC 29544 (= CDC 4562-70).
5. *Enterobacter intermedium* Izard, Gavini, and Leclerc 1980; *Enterobacter* group H<sub>1</sub> (Gavini et al., 1976; Izard et al., 1979). The type strain is strain ATCC 33110 (= CUETM 77-130, CIP 79.27).
6. *Enterobacter amnigenus* Izard et al. 1981; *Enterobacter* group H<sub>3</sub> (Gavini et al., 1976). The type strain is strain ATCC 33072 (= CUETM 77-118).
7. *Enterobacter cancerogenus* (Urosevic) Dickey and Zumoff 1988; *Erwinia cancerogena* Urosevic, 1966. The type strain is ATCC 33241 (= NCPPB 2176). *Enterobacter taylorae* Farmer et al. 1985 (type strain, ATCC 35317 = CDC 2126-81) was found to be a junior synonym of *E. cancerogenus* (Grimont and Ageron, 1989).
8. *Enterobacter asburiae* Brenner et al. 1986; enteric group 17 (Farmer et al., 1985a). The type strain is ATCC 35953 (= CDC 1497-78).
9. *Enterobacter nimipressuralis* (Carter) Brenner et al. 1986; *Erwinia nimipressuralis* Carter 1945. The type strain is strain ATCC 9912.
10. *Enterobacter dissolvens* (Rosen) Brenner et al. 1986; "*Pseudomonas dissolvens*" Rosen 1922; "*Aerobacter dissolvens*" (Rosen) Waldee 1945; *Erwinia dissolvens* (Rosen) Burkholder 1948. The type strain is ATCC 23373. The type strain belongs in the *Enterobacter cloacae* complex.
11. *Enterobacter agglomerans* (Beyerinck 1888) Ewing and Fife 1972; "*Bacillus agglomerans*" Beijerinck 1888; *Erwinia lathyri* (Manns and Taubenhaus) Magrou 1937; *Erwinia herbicola* (Löhnis) Dye 1964; *Erwinia milletiae* (Kawakami and Yoshida) Magrou 1937; *Pantoea agglomerans* (Beijerinck) Gavini et al. 1989. The type strain is ATCC 27155 (= CDC 1461-67, CIP 57.51).
12. *Enterobacter hormaechei* O'Hara et al. 1989; Enteric group 75 (O'Hara et al., 1989). The type strain is ATCC 49162.

#### Remaining Taxonomic Problems in the Genus *Enterobacter*

DNA-relatedness studies of some early described species in the genus *Enterobacter* (*E. aerogenes*, *E. agglomerans*, and *E. cloacae*) unveiled some previously unsuspected problems in the placement of these species.

PRECISE POSITION OF *E. AEROGENES* WITH RESPECT TO THE GENUS *KLEBSIELLA* By DNA hybridization, *E. aerogenes* is closer to *Klebsiella pneumoniae* (about 55% relatedness) than to *E. cloacae* (about 45% relatedness) (Brenner et al., 1972; Steigerwalt et al., 1976). In a numerical taxonomy study (Bascomb et al., 1971), *E. aerogenes* was so close to the genus *Klebsiella* that transfer of this species to the genus *Klebsiella* was proposed. Since the name "*K. aerogenes*" had been used for bacteria which are undistinguishable from *K. pneumoniae* by DNA hybridization, the name *K. mobilis* was proposed for *E. aerogenes* (Bascomb et al., 1971). Carbon source utilization patterns of *E. aerogenes* are also closer to those of *Klebsiella* species than to those of *Enterobacter* species (P.A.D. Grimont, unpublished observations). In the authors' opinion, *E. aerogenes* can reasonably be placed in the genus *Klebsiella*. The problem is only a nomenclatural one.

HETEROGENEITY OF *ENTEROBACTER AGGLOMERANS* *E. agglomerans* comprises a very complex group of environmental bacteria which may cause opportunistic infections. The name covers many



Table 1. Correspondence between phenons and DNA-relatedness groups in the *Enterobacter agglomerans* complex..

Phenon (Gavini et al., 1983)	Phenon (Verdonck et al., 1987)	DNA group (Brenner et al., 1984)	Nomenspecies
(B4)*	7B	V	—
(B4)	8	XIII	<i>Pantoea agglomerans</i> ( <i>Erwinia agglomerans</i> , <i>E. herbicola</i> , <i>E. milletiae</i> )
(B5)	9	II	—
(B5)	10	III	<i>Pantoea dispersa</i>
(B9)	11	—	—
—	Ungrouped	X	—
B8	12	VI	<i>Erwinia ananas</i> , <i>E. uredovora</i>
Ungrouped	16	XII	—
C	(17)	—	<i>Rahnella aquatilis</i>
D1	(17)	—	—
Ungrouped	18	IX	—
(B9)	23	VIII	—
E2, E3, E5	(26)	XI	<i>Leclercia adecarboxylata</i>
E4	(26)	VII	—
—	30	IV	—

\*Parentheses indicate partial correspondence only.

(20 to 40 or more) genomic groups (Brenner et al., 1984) or phenons (Gavini et al., 1983; Verdonck et al., 1987). In addition to this diversity, strains of the *E. agglomerans* complex are not closely related to *E. cloacae* (the type species of the genus *Enterobacter*) by DNA hybridization. Some groups in this complex have been individualized as new genera (*Rahnella aquatilis*, *Ewingella americana*, *Leclercia adecarboxylata*). There apparently is confusion in the literature, and a close examination of published papers for the presence of commonly studied strains is needed to extract convergent pieces of information.

Table 1 lists the phenons and DNA groups that have been proposed. The numerical study of Gavini et al. (1983), based on 169 strains, yielded five phenons (A to E). Phenon A corresponded to *Erwinia carotovora*. Phenon B, which included strains of the *Enterobacter agglomerans* complex, was split into nine smaller phenons (B1 to B9). Phenon B4 contained the type strains of *Erwinia herbicola* and *Erwinia milletiae*. Phenon B8 contained the type strain of *Erwinia ananas* and a reference strain of *Erwinia uredovora*. Phenon C corresponded to *Rahnella aquatilis*. Phenon D was split into three smaller phenons (D1 to D3), phenon D2 corresponding to *Enterobacter sakazakii*. Phenon E was split into five smaller phenons (E1 to E5), phenon E5 containing strains previously identified as *Escherichia adecarboxylata*. A larger numerical study (Verdonck et al., 1987), based on 529 strains including many type and reference strains, led to the distribution of 66 strains of the *Enterobacter agglomerans* complex into 21 phenons. The correspondence between phenons from both studies (Gavini et al., 1983 and Verdonck et al.,

1987) is given in Table 1. The DNA-relatedness work of Brenner et al. (1984) showed the extreme genomic diversity of the *Enterobacter agglomerans* complex since, of 124 strains studied, 90 fell into 13 DNA groups (I to XIII), and 34 strains did not fit into any group. Furthermore, four groups (V, XI, XII, and XIII) were heterogeneous with respect to the thermal stability of heteroduplexes ( $\Delta T_m$  values). An interesting finding was that aerogenic (gas-producing) strains and anaerogenic strains were not found in the same hybridization group.

By DNA hybridization, Lind and Ursing (1986) identified 52 of 86 clinical isolates with *Enterobacter agglomerans* sensu stricto. In the same study, they demonstrated the synonymy of *Enterobacter agglomerans*, *Erwinia herbicola*, and *Erwinia milletiae*. This synonymy was confirmed by Beji et al. (1988) who, in addition, identified DNA group XIII (Brenner et al., 1984) with *Enterobacter agglomerans* sensu stricto.

To separate *Enterobacter agglomerans*/*Erwinia herbicola* from the genera *Enterobacter* and *Erwinia*, the new genus *Pantoea* has been proposed, with *P. agglomerans* as type species (Gavini et al., 1989). Strains of DNA group III (Brenner et al., 1984) or phenon 8 (Verdonck et al., 1987) were proposed as the new species *Pantoea dispersa* (Gavini et al., 1989). The DNA groups closest to *Enterobacter agglomerans* (*Pantoea agglomerans*) are DNA groups II, III, IV, V, and VI (Lind and Ursing, 1986). It is interesting that strains of group II to VI and XIII are characterized by the presence of a glucose oxidation pathway producing 2-ketogluconate from glucose (Bouvet et al., 1989). This finding has been extended to DNA group I (P.A.D. Grimont,

unpublished observations). Thus, the genus *Pantoea* can be envisioned to include DNA groups I, II, IV, V, and VI ("*Pantoea ananas*"), in addition to groups XIII (*P. agglomerans*) and III (*P. dispersa*).

DNA group XI (Brenner et al., 1984) was identified with *Escherichia adecarboxylata* by DNA hybridization (Izard et al., 1985). *Escherichia adecarboxylata* was recently transferred to a new genus *Leclercia* as *L. adecarboxylata* (Tamura et al., 1986). DNA group VII (Brenner et al., 1984) was found close to (but distinct from) *Escherichia (Leclercia) adecarboxylata* (Izard et al., 1985). In our laboratory, strains of DNA group VII were found indistinguishable from strains of phenon E4 (Gavini et al., 1983) by carbon source utilization tests (P.A.D. Grimont, unpublished observations). Thus, DNA group VII is a good candidate as a new species of *Leclercia*.

DNA group VIII (Brenner et al., 1984) was found to be 64% related to *Erwinia persicinus* (a close relative of *Erwinia rhapontici*), and in a numerical taxonomy study, DNA group IX (Brenner et al., 1984) was close to *Rahnella aquatilis* (Verdonck et al., 1987).

More work is needed to characterize all the species in the *E. agglomerans* complex and to assign them to *Pantoea*, *Leclercia*, and related genera.

#### HETEROGENEITY OF *ENTEROBACTER CLOACAE*

Early DNA-relatedness studies on *E. cloacae* (Steigerwalt et al., 1976) showed the genomic heterogeneity of this nomenspecies. In work done in our laboratory (P.A.D. Grimont, unpublished observations) on 49 strains previously identified as *E. cloacae*, 45 strains fell into five DNA relatedness groups (1 to 5). Group 1 contained only three strains, including the type strains of *E. cloacae* and *E. dissolvens*. Reassociated DNA from these type strains showed some divergence ( $\Delta T_m$  values of 4.5°C). In spite of a search for similar strains in our large laboratory collection, no other strain of group 1 could be found. Group 2 contained seven strains, including reference strain CDC 1347-71 (Steigerwalt et al., 1976). Group 3 contained 15 strains including the type strain of *Enterobacter hormaechei*. This group was slightly heterogeneous with  $\Delta T_m$  values ranging from 0.0 to 4.0°C. Group 4 contained 17 strains and could be split into three subgroups on the basis of  $\Delta T_m$  values (0.0 to 2.5°C within subgroups and 4.0 to 6.5°C between subgroups). Subgroup 4a contained the type strain of *Enterobacter asburiae*. Group 5 (earlier referred to as group 6 [Bouvet et al., 1989]) contained three nitrogen-fixing strains.

The three following problems are raised by this unpublished study. The first problem deals

with *E. hormaechei*. A group of similar strains (enteric group 75), found to differ phenotypically from *E. cloacae* was shown to be genomically distinct from the type strain of *E. cloacae* and thus described as a new species, *E. hormaechei* (O'Hara et al., 1989). In our group 3, we found only one strain phenotypically similar to *E. hormaechei*. All other strains of group 3 fitted the classical definition of *E. cloacae* (Richard, 1984). Group 3 strains (including *E. hormaechei*) were found to produce an active glucose dehydrogenase, and this property differentiates group 3 from the other groups in the *E. cloacae* complex (Bouvet et al., 1989 and P.A.D. Grimont, unpublished observations). Group 3 should be named *E. hormaechei*, and the species definition should be amended.

The second problem deals with *E. asburiae*. Enteric group 17 differed from *E. cloacae* by some phenotypic features and DNA hybridization showed enteric group 17 (*E. asburiae*) to be distinct from the type strain of *E. cloacae*. Our subgroup 4a is genomically and phenotypically identical with *E. asburiae*. However, subgroups 4b and 4c are less differentiable from *E. cloacae* by phenotypic characteristics. Group 4 (including subgroups 4a, 4b, and 4c) should be called *E. asburiae* and the species definition should be amended.

The third problem is in fact the cause of the other two. The choice of the neotype strain for *E. cloacae* was unfortunate. This strain was isolated from cerebrospinal fluid, which is not the usual habitat of what is currently known as *E. cloacae*. Either the nomenclatural rules should be observed, and *E. cloacae* (our group 1) will disappear from routine clinical laboratory work (because most strains presently labeled *E. cloacae* are in fact *E. hormaechei* or *E. asburiae*), or the type strain is changed for a strain from our group 2 (which can be encountered in clinical microbiology, although less frequently than *E. hormaechei* and *E. asburiae* genomic species).

## Ecology and Epidemiology

Ecological information about the *Enterobacter* species is clouded by uncertainties relative to the delineation and recognition of species within the *E. agglomerans* and *E. cloacae* complexes so that current ideas of ecology and epidemiology given below may become inaccurate when the above-mentioned complexes are resolved into many species.

*Enterobacter* species are found in the natural environment in habitats such as water, sewage, vegetables, and soil. Before the widespread use of antibiotics, *Enterobacter* species were rarely found as pathogens, but these organisms are now

increasingly encountered, causing nosocomial infections such as urinary tract infections and bacteremia (reference is not an exact match Eichhoff et al., 1966). In addition, they occasionally cause community-acquired infections. In 1975 in the United States, *Enterobacter* species accounted for 4.6% of all pathogens causing infections and accounted for 5.7% of all cases of primary bacteremia (Center for Disease Control, 1977). In 1984, *Enterobacter* species accounted for 5.9% of all nosocomial infections in U.S. hospitals and 6.3% of all nosocomial bacteremia (Centers for Disease Control, 1984).

Older reports on *Klebsiella-Enterobacter* infections were taxonomically imprecise. Some laboratories used one name (*Enterobacter*) for an organism isolated from urine and another name (*Klebsiella*) for the identical organism isolated from sputum. Other laboratories based the identification on the colony type: mucoid colonies were designated *Klebsiella* and nonmucoid colonies were reported as *Enterobacter* (Brenner, 1981).

*E. cloacae* occurs as a commensal in water, sewage, soil, meat, hospital environments, the skin, and in the intestinal tracts of humans and animals. Among 234 patients, the rate of stool carriage of *E. cloacae* on admission to the hospital was 2.6%. This rate increased to 4% after antibiotic therapy (Rose and Schreier, 1968). In a large European study, *E. cloacae* was the most prevalent Gram-negative isolate colonizing the feces of the leukemic patients (van der Waaig et al., 1977). Rosenthal and Tager (1975) found isolates of *E. cloacae* in the pharynx of three of 29 normal persons.

*E. cloacae* infections are endemic in some hospitals. In 1966, this organism tended to colonize rather than infect hospitalized patients (Rose and Koch, 1966). At Boston City Hospital, *E. cloacae* was responsible for a variety of nosocomial and, occasionally, community-acquired infections. This species was more prevalent than *E. aerogenes* (Dans et al., 1970). In a study of infections in patients with nonlymphocytic leukemia, *E. cloacae* produced two of 87 nosocomial infections and one of 44 cases of bacteremia (Alexander, 1979). *E. cloacae* has been found in a variety of infections such as endocarditis, ventriculitis, meningitis, arthritis or osteomyelitis, urinary tract infections, and pneumonia (reviewed by John et al., 1982). *E. cloacae* bacteremia was first reported at Boston City Hospital (Maiztegui et al., 1965). Working at a large metropolitan medical center, Fuchs (1979) found isolates of *E. cloacae* in 1.8% of postoperative wound infections, in 2.9% of respiratory infections, and in 2.2% of urinary tract infections, but none among 163 blood infections. At the Manhattan Veterans Hospital in 1973–1974, of 17 bacteremic patients,

six were infected with gentamycin-resistant *E. cloacae*, and two of the six died (Richmond et al., 1975). This species has been endemic at a medical center hospital and caused 4.5% of all cases of bacteremia in 1978 (John et al., 1982). *E. cloacae* bacteremia occurs in burn units. Patients often have multiple sites (urine, wound, sputum) colonized before bacteremia develops (John et al., 1982). In a study of 41 patients burnt in military action, *Enterobacter* was the fifth most common organism colonizing or infecting burn wounds (Sidi et al., 1977). In a recent study, *E. cloacae* accounted for 29 of 58 episodes of *Enterobacter* bacteremia and 24 of 42 nosocomial *Enterobacter* bacteremia, and the most common portal of entry was the lungs (Watanakunakorn and Weber, 1989). *E. cloacae* was spread by contaminated hands of personnel and by cross-contamination of hydrotherapy water in an outbreak of infections which occurred in a burn center (Mayhall et al., 1979). Heavily colonized stools of premature infants were the source of one epidemic (Powell et al., 1980).

Contaminated medicinal agents can be sources of outbreaks. Maki and Martin (1975) showed that *E. cloacae*, *E. agglomerans*, and *Serratia marcescens* multiplied in 5% dextrose solution at 25°C better than did other members of the Enterobacteriaceae. *E. cloacae* has produced epidemics associated with contaminated benzalkonium chloride (Malizia et al., 1960), ice (Newson, 1968), platelets (Buchholz et al., 1970), pressure monitoring devices (Weinstein et al., 1976; Borobio and Perea, 1975), and hemodialysis (Buxton et al., 1978). Maki et al. (1976) reported on a nationwide epidemic in the United States caused by contaminated intravenous fluid, which caused 163 cases of *E. cloacae* bacteremia in 25 hospitals, with a mortality rate of 7.8%.

*E. aerogenes* occurs in water, sewage, soil, dairy products, and the feces of humans and animals. Platt et al. (1976) described the prevalence of *E. aerogenes* strains in the preputial flora of healthy stallions. Among 234 patients, the rate of stool carriage of *E. aerogenes* on admission to the hospital was 3.4%. This rate increased to 6% after antibiotic therapy (Rose and Schreier, 1968).

*E. aerogenes* produces infections and bacteremia in both hospitalized and nonhospitalized patients. In a neurosurgical clinic, *E. aerogenes* caused 9.8% of infections in 1972 (John et al., 1982). *E. aerogenes* bacteremia was associated with contaminated intravenous fluid in pediatric patients (Edwards et al., 1978). In an Ohio hospital, *E. aerogenes* accounted for 25 of 58 episodes of bacteremia and 17 of 42 nosocomial bacteremia (Watanakunakorn and Weber, 1989).

The *Enterobacter agglomerans* complex is ubiquitous in the environment. This species pre-

dominates on the leaf and bract of pre- and post-senescent cotton plants (DeLuca and Palmgren, 1986). The main species of Gram-negative bacteria found in cotton dusts in mills is *E. agglomerans* (Haglund et al., 1981). The following occurrences were discussed by Slade and Tiffin (1984). Strains of *Enterobacter agglomerans/Erwinia herbicola* are found on the aerial surfaces of plants and within healthy plant tissues and seeds. Nitrogen-fixing strains have been found in the rhizosphere of wheat and sorghum. In fact, these bacteria are typical of the innermost part of the rhizosphere of wheat (Kleeberger et al., 1983). Strains of this complex have been isolated from water, papermill-process water, soil, and decaying wood. They are frequently isolated from damaged plant tissues and lesions although they are rarely considered to be pathogens. However, a pathogenic role has been shown in some instances. Strains of *Erwinia milletiae* produced  $\beta$ -indolyl acetic acid (a plant hormone), causing galls on some plants in Japan (reviewed by Slade and Tiffin, 1984). Some strains were found to initiate freezing of buffer solutions (which would normally freeze at temperatures below  $-10^{\circ}\text{C}$ ) at about  $-4^{\circ}\text{C}$ . This ice nucleating property plays a critical role in causing frost damage on plants (Kozloff et al., 1983; Lindow et al., 1978). Some strains cause a pink discoloration of canned pineapple (Cho et al., 1980), and others are associated with stalk and leaf necrosis of onion (Hattingh and Walters, 1981). Strains of *Erwinia uredovora* attack the uredia of rust (*Puccinia* spp.) on wheat, oats, and rye (Pon et al., 1954; Dye, 1969).

Strains of the *E. agglomerans* complex have been used for the biological control of plant pathogens (such as *Erwinia amylovora* or *Xanthomonas oryzae*); eliciting their effect either by competition for nutrients, production of acid, or by bacteriocin or phage production (reviewed by Slade and Tiffin, 1984).

*E. agglomerans* may occur in clinical samples (blood, wounds, sputum, urine), often with dubious clinical significance (von Graevenitz, 1970; Gilardi and Bottone, 1971; Pien et al., 1972). In some cases (von Graevenitz and Palermo, 1980; Cooper-Smith and von Graevenitz, 1978), clinical significance has been demonstrated. In an Ohio hospital, *E. agglomerans* accounted for 4 of 58 episodes of *Enterobacter* bacteremia and one of 42 nosocomial bacteremia (Watanakunakorn and Weber, 1989). In 1970, *E. agglomerans* was implicated in a nationwide outbreak of septicemia caused by contaminated closures on bottles of infusion fluids in the United States. Twenty-five hospitals were involved, with 378 cases and 40 deaths (Maki et al., 1976).

The natural habitat of *E. sakazakii* is unknown. Unless thorough phenotypic descrip-

tions are given in the literature (as yellow-pigmented *E. cloacae*), *E. sakazakii* can hardly be traced in older clinical reports. The yellow pigment production, a distinguishing character (best seen at  $25^{\circ}\text{C}$ ), is greatly decreased at the usual incubation temperature of  $37^{\circ}\text{C}$ . Urmenyi and Franklin (1961) reported two cases of neonatal meningitis in England due to yellow-pigmented *E. cloacae*. This organism was isolated from brain tissue and cerebrospinal fluid. Other cases of meningitis have also been described (Jøker et al., 1965; Adamson and Rogers, 1981; Muytjens et al., 1983). Four isolates from wounds and urine were described by Gilardi and Bottone in 1971. This organism was responsible for a necrotizing meningoencephalitis complicated by ventricular compartmentalization and abscess formation (Kleiman et al., 1981). Monroe and Tift (1979) reported neonatal bacteremia in the absence of meningitis.

*Enterobacter dissolvens* and *E. nimipressuralis* have not been observed in clinical specimens and have only been recovered from environmental sources. *E. dissolvens* was first isolated by Rosen (1922) from diseased corn and is found in rotting cornstalks. *E. nimipressuralis* was described as being the causal agent of wetwood in elm trees (Carter, 1945). These two organisms are very close phenotypically to *E. cloacae* and it is possible that any clinical isolates would have been identified as *E. cloacae*.

*E. intermedium* was isolated from surface water and unpolluted soil (Izard et al., 1980). Prats et al. (1987) reported the isolation of *E. intermedium* from foot suppuration, blood, feces and bile.

*E. amnigenus* was mostly isolated from water, but some strains were isolated from clinical specimens such as respiratory tract, wound, or feces (Farmer et al., 1985a).

*E. taylora* (*E. cancerogenus*) strains were isolated from blood and spinal fluid (Farmer et al., 1985b). Westblom and Coggins (1987) reported the first case of osteomyelitis caused by this species.

*E. gergoviae* was found in a variety of environmental sources, such as water, cosmetics, and clinical sources, in France, Africa, and the United States (Richard et al., 1976; Brenner et al., 1980). Multiply drug-resistant strains were found in urine samples during an infection outbreak (Richards et al., 1976).

*E. asburiae* strains were isolated from clinical specimens, mostly urine, respiratory tract, feces, wounds, and blood (Brenner et al., 1986). The clinical significance of this organism is not known.

*E. hormaechei* was isolated from wounds, sputum, and blood (O'Hara et al., 1989).

## Properties Relevant to Pathogenicity for Humans

Histamine decarboxylases are responsible for histamine formation from histidine in food products, thus occasionally resulting in histamine poisoning in humans. Histidine decarboxylase from *E. aerogenes* has been purified and characterized. The enzyme requires pyridoxal-5'-phosphate as a coenzyme and decarboxylates histidine only among the amino acids derived from protein (Guirard and Snell, 1987).

Adhesive properties may be important in the establishment or maintenance of bacterial infections. Adhesins are often also hemagglutinins (HA) and may or may not be located on fimbriae. Most strains of *Enterobacter amnigenus*, *E. cloacae*, and *E. sakazaki* produce a mannose-sensitive hemagglutinin (MS-HA) associated with type 1 fimbriae, i.e., thick, channelled fimbriae of external diameter 7 to 8 nm. These fimbriae can be coated by type 1 fimbrial antiserum against *E. cloacae* 035 but not by type 1 fimbrial antiserum against *Klebsiella pneumoniae* K55/1 (Adegbola and Old, 1983). No other hemagglutinin and fimbrial type has been observed in these species.

Seven of eight *E. gergoviae* strains were found to produce a mannose-resistant *Klebsiella*-like HA (MR/K-HA) agglutinating tanned ox erythrocytes, and they were associated with type 3 fimbriae, i.e., thin, nonchannelled fimbriae of external diameter 4 to 5 nm. These fimbriae can be coated with type 3 fimbrial antiserum against *K. oxytoca* K70/1 (Adegbola and Old, 1983). No other hemagglutinin and fimbrial type has been observed in this species.

Six of eight *E. intermedium* strains produced MS-HA or MR/K-HA or both. MS-HA was associated with type 1 fimbriae that could not be coated by type 1 fimbrial antisera against either *E. cloacae* 035 or *K. pneumoniae* K55/1 (Adegbola and Old, 1983). MR/K-HA produced by *E. intermedium* was associated with type 3 fimbriae that could be coated by type 3 fimbrial antiserum against *Serratia* but not by that against *E. cloacae* or *K. pneumoniae* (Adegbola and Old, 1983; Old and Adegbola, 1984).

All strains of *E. aerogenes* tested produced hemagglutinins (Adegbola and Old, 1983; Adegbola and Old, 1985). Most strains produced a mannose-sensitive hemagglutinin that seemed to be associated with thin (4-nm), nonchannelled fimbriae. These thin fimbriae were antigenically different from the thin fimbriae of other *Enterobacter* and *Klebsiella* species (Adegbola and Old, 1985).

Most strains also produced a nonfimbrial mannose-resistant *Proteus*-like hemagglutinin

(MR/P-HA) which agglutinated untanned guinea pig and horse erythrocytes. A single strain (NCIMB 11460) produced a mannose-resistant *Klebsiella*-like hemagglutinin associated with type-3 fimbriae antigenically similar to *Klebsiella* type-3 fimbriae (Adegbola and Old, 1985).

A single strain of *E. agglomerans* was found to be devoid of hemagglutinin (Adegbola and Old, 1983).

Iron is essential for bacterial growth. In the human body, iron is complexed to carrier molecules such as transferrin (in the serum) or lactoferrin (in milk and other secretions), or sequestered within cells (in heme proteins). When growing under iron-limiting conditions, potentially pathogenic Enterobacteriaceae produce high-affinity systems (siderophores) to solubilize and import the required iron. The iron-chelating compounds produced are mostly of two sorts, phenolates (e.g., enterochelin) and hydroxamates (aerobactin) (Payne, 1988). All strains of *E. cloacae*, *E. aerogenes*, *E. gergoviae*, *E. sakazaki*, and *E. agglomerans* tested by Reissbrodt and Rabsch (1988) produced enterochelin. Some strains of all these species except *E. agglomerans* were found to produce aerobactin. Two of nine strains of *E. agglomerans* produced a hydroxamate compound other than aerobactin (Reissbrodt and Rabsch, 1988). Aerobactin was first isolated from a strain of *E. aerogenes* (then called "*Aerobacter aerogenes*") (Gibson and Magrath, 1969). Aerobactin and cloacin DF13 bind to the same receptor sites located in the outer membrane (Van Tiel-Menkveld et al., 1982). Aerobactin is encoded by a relatively simple genetic system, which has been extensively characterized. Only four genes are required for synthesis of aerobactin (Carbonetti and Williams, 1984). *E. cloacae* harbors a relatively large conjugative plasmid coding for susceptibility to cloacin DF13 as well as for production and uptake of aerobactin (Krone et al., 1985).

The lipopolysaccharide from *E. agglomerans* (commonly found in cotton dust) can bind to the pulmonary lipid-proteinaceous lining material (surfactant) and alter its surface tension properties (DeLucca et al., 1988). This binding in the lung may change the physiological properties of surfactant and be a possible mechanism for the pathogenesis of byssinosis, an occupational respiratory disorder caused by the inhalation of cotton dust (DeLucca et al., 1988).

### Antimicrobial Susceptibility

Strains of the *Enterobacter cloacae* complex (including *E. asburiae* and *E. hormaechei*) that have not acquired any resistance determinant are naturally resistant to aminopenicillins (ampi-

cillin) and first-generation cephalosporins (cephalothin). These strains are sensitive to carboxypenicillins (carbenicillin). Among the second-generation cephalosporins, cefamandole is moderately active whereas cefoxitin is not. Third-generation cephalosporins (e.g., cefotaxime, moxalactam) are most active. This natural pattern of resistance is due to the production of an inducible chromosomal cephalosporinase. When a cefoxitin disk is placed in the vicinity of a cefotaxime disk on an antibiotic susceptibility test plate, the wide inhibition zone around the cefotaxime disk is narrower in the vicinity of the cefoxitin disk (Jarlier, 1985).

Acquired resistance arises from the production of a plasmid-determined penicillinase that inactivates carboxypenicillins (carbenicillin) and often ureidopenicillins (mezlocillin) but that does not inactivate third-generation cephalosporins. However, resistance to third-generation cephalosporins (cefotaxime) has been observed, due to the derepression of chromosomal cephalosporinase, or to a plasmid-mediated, extended-spectrum  $\beta$ -lactamase (Jacoby, 1990), or to a reduced expression of outer membrane proteins such as porins (Aggeler et al., 1987). *N*-formimidoyl thienamycin (imipenem) remains active on cefotaxime-resistant strains).

Susceptibility to chloramphenicol, nalidixic acid, and tetracycline differs for *E. cloacae* isolated in different hospitals.

In 1970, all strains of *E. cloacae* were susceptible to gentamicin. During the mid-1970s, plasmid-controlled aminoglycoside resistance spread among Gram-negative bacilli worldwide, and resistance to gentamicin, tobramycin, and (to a lesser extent) amikacin became a concern (John et al., 1982). Resistance of *E. cloacae* to diverse aminoglycoside antibiotics is due to the production of aminoglycoside-modifying enzymes such as aminoglycoside-2''-*O*-nucleotidyltransferase, aminoglycoside-3'-*O*-phosphotransferase, aminoglycoside-3-*N*-acetyltransferase, or aminoglycoside-6'-*N*-acetyltransferase (John et al., 1982).

Antibiotic susceptibility of *E. aerogenes* is similar to that of *E. cloacae*. Most strains of *E. aerogenes* are naturally resistant to ampicillin, cephalothin, and cefoxitin, and susceptible to carbenicillin.

Generally *E. sakazakii* is susceptible to ampicillin, carbenicillin, amikacin, cefamandole, cefoxitin, aminoglycosides, chloramphenicol, and nalidixic acid, but 87% of the strains are resistant to cephalothin (Farmer et al., 1980). Compared to *E. cloacae*, *E. sakazakii* strains exhibit larger zones of inhibition around ampicillin and cephalothin antibiotic disks, a property which also helps to differentiate the latter species from *E. cloacae*.

Most *E. agglomerans* strains are naturally resistant to ampicillin and cephalothin and susceptible to many antibiotics including aminoglycosides, carbenicillin, cefamandole, cefuroxime, and cefoxitin. Resistance to carbenicillin may occur.

*E. gergoviae* strains are susceptible to antibiotics but strains isolated from urinary tract infection were found to be resistant to ampicillin and cephalothin (Richard et al., 1976).

Strains of *E. taylorae* (= *E. cancerogenus*) were generally susceptible to colistin, nalidixic acid, sulfadiazine, gentamicin, kanamycin, chloramphenicol, tetracycline, cefotaxime, cefoperazone, piperacillin, ticarcillin, tobramycin, and gentamicin, and resistant to cephalothin and cefadroxil. Susceptibility to ampicillin and carbenicillin varies among strains (Westblom and Coggins, 1987).

Except for ampicillin, cephalothin, and cefoxitin, *E. intermedium* is susceptible to all the antimicrobial agents generally active against Gram-negative rods.

## Biotechnology

### Enzymes

Diacetyl is an undesirable flavor component in young beer.  $\alpha$ -Acetolactate (an intermediate of the isoleucine-valine pathway) is converted to diacetyl solely by non-enzyme-catalyzed oxidative decarboxylation, and then it is reduced, during the maturation process, to acetoin by diacetylreductase.  $\alpha$ -Acetolactate can be converted directly to acetoin by an  $\alpha$ -acetolactate decarboxylase which is found in several *Enterobacter* species. The  $\alpha$ -acetolactate decarboxylase gene of *E. aerogenes* has been cloned (Sone et al., 1987) and introduced by transformation into brewer's yeast. The diacetyl concentration in wort fermented by the recombinant yeast was lower than that in wort fermented by the parental strain (Sone et al., 1988).

Chromate (hexavalent chromium) is a strong oxidant that is toxic and mutagenic in bacterial test systems. A chromate-resistant strain of *E. cloacae* that reduces chromate to chromium hydroxide (insoluble and less toxic) under anaerobic conditions has been isolated and characterized (Wang et al., 1989).

### Nitrogen-Fixing Species of *Enterobacter*

Nitrogen-fixing *Enterobacter* species have been occasionally reported (Barroquio and Watanabe, 1981) but the confusion regarding the taxonomy of *E. cloacae* and *E. agglomerans* render published identifications as provisional.

The specific nitrogenase activity of *E. agglomerans* isolated from paper-mill-process water was

10  $\mu\text{mol}$  of acetylene per mg protein per h (Neilson and Sparell, 1976) and that reported for *E. agglomerans* isolated from grass root was only 1.6  $\mu\text{mol}$  of acetylene per mg protein per h (Haahtela et al., 1983).

Nitrogen-fixing *E. cloacae* have been isolated from the roots of dryland and wetland rices (Ladha et al., 1983). The nitrogen-fixing *E. cloacae* strains isolated by Bally et al. (1983) were found to belong in *E. cloacae* genomic group 5 (P.A.D. Grimont, unpublished observations).

## Isolation

Media used in the isolation of *Enterobacter* species are similar to those used for other Enterobacteriaceae. There are no selective media for *Enterobacter* species. The requirements of a selective medium are 1) a precise and stable delineation of the genus (or species, if the medium is to be species specific); 2) known common properties within the genus (or species) which are uncommon outside the genus (or species); and 3) a clinical, public health, or special need for such selective medium. Due to pending changes in the delineation of the genus *Enterobacter* (e.g., exclusion of the *E. agglomerans* complex) and of the species *E. cloacae* and *E. agglomerans*, conditions 1 and 2 are not met. Except for some epidemiological studies, the selective isolation of *Enterobacter* spp. is rarely necessary since the presence of *Enterobacter* spp. in pluribacterial habitats (feces, throat, skin) is clinically meaningless. The public health significance of *Enterobacter* spp. in water or foods is uncertain. Isolation of *Enterobacter* spp. from clinical specimens is done either by direct plating on blood agar, tryptic soy agar, or nutrient agar (e.g., pus, urine) or by plating after prior growth in tryptic soy broth or nutrient broth (blood, pus, cerebrospinal fluid).

On nutrient agar, *E. cloacae*, *E. aerogenes*, *E. gergoviae*, *E. amnigenus*, *E. nimipressuralis*, *E. intermedium*, or aerogenic *E. agglomerans* strains (*Leclercia* spp.) form colonies that are round, 2–3 mm in diameter, and slightly iridescent or flat with irregular edges (Richard, 1984). Aerogenic *E. agglomerans* strains can form yellow-pigmented colonies. Anaerogenic *E. agglomerans* strains (*Pantoea* spp.) form either mucoid colonies, or smooth and irregularly round colonies, or rough and wrinkled colonies, which are difficult to remove with a platinum wire. These colonies are often yellow pigmented. *E. sakazakii* strains form bright yellow colonies at 25°C or pale yellow colonies at 37°C, 1 to 3 mm in diameter. These colonies are smooth, mucoid, or dry (Farmer et al., 1980).

Differential media which are not inhibitory for the Enterobacteriaceae are often used. These are bromothymol blue-lactose agar, phenol red-lactose agar, Drigalski lactose agar, eosin methylene blue agar, or MacConkey agar. It should be remembered that some strains of a species known to produce acid from lactose may fail to do so. A differential medium, lysine-ornithine-mannitol agar containing vancomycin, was proposed for the isolation of *Enterobacter agglomerans*. It yields colorless colonies from mannitol-negative strains, yellow colonies from mannitol-positive, ornithine- and lysine-decarboxylase negative strains, and greenish-blue colonies from mannitol-positive strains producing one or both decarboxylases (Bucher and von Graevenitz, 1982).

## Identification

The normal methods used for the identification of *Enterobacter* species and other Enterobacteriaceae can be found in Ewing (1986) and Farmer et al. (1980). However, other tests are extremely useful in enterobacterial taxonomy. These include carbon source utilization tests, glucose oxidation (in the presence or in the absence of added pyrroloquinoline quinone), gluconate and 2-ketogluconate dehydrogenase tests, and tetrathionate reductase and  $\beta$ -xylosidase tests. These tests and a modified Voges-Proskauer test are described in detail in The Genus *Serratia* from the second edition.

### Conditions of Incubation

Best results are obtained when *Enterobacter* cultures are incubated at 30°C. At 37°C, yellow pigmentation of *E. sakazakii* or *E. agglomerans* may be weak.

### Identification of *Enterobacter* at the Genus Level

The impending changes in the delineation of the genus *Enterobacter* prevent any stable definition of the genus. Presently, most *Enterobacter* species and biogroups are positive for the Voges-Proskauer and Simmons citrate tests, are motile, produce acid from D-glucose, D-mannitol, salicin, L-arabinose, L-rhamnose, D-xylose, trehalose, D-cellobiose, and maltose; hydrolyze *o*-nitrophenyl- $\beta$ -D-galactoside, reduce nitrate, and oxidize D-glucose to D-gluconate in the presence of pyrroloquinoline quinone. All *Enterobacter* species (except some *E. agglomerans* genomic groups) produce gas from glucose. The following substrates are utilized by most strains: *N*-acetyl-D-glucosamine, D-alanine, L-alanine, L-arabinose, L-aspartate, D-cellobiose, citrate,

D-fructose, fumarate, D-galactose, D-galacturonate, gentiobiose, D-gluconate, D-glucosamine, D-glucose, D-glucuronate, L-glutamate, DL-glycerate, glycerol, 2-ketogluconate, DL-lactate, lactose, maltose, maltotriose, D-mannitol, D-mannose, 1-O-methyl- $\beta$ -D-galactoside, 1-O-methyl- $\beta$ -D-glucoside, L-rhamnose, D-ribose, L-serine, succinate, D-trehalose, and D-xylose.

Most *Enterobacter* species or biogroups are negative for the following tests: H<sub>2</sub>S production from thiosulfate, phenylalanine deaminase, tetrathionate reduction, tributyrin or corn oil hydrolysis,  $\beta$ -glucuronidase, and 2-ketogluconate dehydrogenase. Most strains cannot utilize the following substrates as sole carbon and energy sources: caprate, caprylate, ethanolamine, glutarate, itaconate, D-melezitose, 3-phenylpropionate, propionate, and L-sorbose.

### Identification of *Enterobacter* at the Species Level

The characteristics that are most useful for the identification of seven *Enterobacter* species and the *E. cloacae* and *E. agglomerans* complexes are given in Table 2 and detailed characteristics are given in Tables 3, 4, 5. Results of carbon sources utilization tests given in Tables 2–5 are mostly unpublished observations.

*E. aerogenes* and *E. gergoviae* possess gluconate dehydrogenase and lysine decarboxylase, produce acid from D-arabitol, and utilize D-arabitol and histamine, and most strains utilize gentisate, 3-hydroxybenzoate, and tricarballylate. *E. aerogenes* can be separated from *E. gergoviae*

by urea hydrolysis,  $\beta$ -xylosidase test, acid production from adonitol, methyl- $\alpha$ -D-glucoside, and sorbitol; and by utilization of *trans*-aconitate, adonitol, *m*-coumarate, maltitol, mucate, palatinose, D-saccharate, and D-sorbitol.

*E. intermedium* is positive for ornithine decarboxylase and acid production from dulcitol, and utilization of dulcitol, 4-hydroxybenzoate, 5-ketogluconate, protocatechuate, quinate, D-sorbitol, and D-tagatose. It is negative for lysine decarboxylase and arginine dihydrolase.

*E. sakazakii*, *E. cancerogenus* (= *E. taylora*), *E. amnigenus*, *E. nimipressuralis*, and the *E. cloacae* complex are positive for arginine dihydrolase and ornithine decarboxylase, and negative for lysine decarboxylase and gluconate dehydrogenase. These species can be separated by the following tests: yellow pigment production, glucose dehydrogenase (without added pyrroloquinoline quinone),  $\beta$ -xylosidase, acid production from D-raffinose, D-sorbitol, and sucrose, and utilization of L-fucose, D-lyxose, maltitol, D-melibiose, 3-O-methyl-D-glucose, palatinose, phenylacetate, putrescine, D-sorbitol, and sucrose. In addition, an  $\alpha$ -glucosidase test was proposed to separate *E. sakazakii* ( $\alpha$ -glucosidase positive) from other *Enterobacter* species ( $\alpha$ -glucosidase negative) (Muytjens et al., 1984).

The phenotypic properties of genomic groups and subgroups within the *E. cloacae* complex are given in Table 3. Genomic group 1 is presently only represented by three strains, including the type strains of *E. cloacae* and *E. dissolvens*. This group is difficult to differentiate phenotypically

Table 2. Differentiation of *Enterobacter* species.

Test	<i>E. aerogenes</i>	<i>E. gergoviae</i>	<i>E. sakazakii</i>	<i>E. intermedium</i>	<i>E. cancerogenus</i>	<i>E. amnigenus</i>	<i>E. nimipressuralis</i>	<i>E. cloacae</i> complex	<i>E. agglomerans</i> complex
Yellow pigment	-	-	+	-	-	-	-	-	v
Glucose dehydrogenase	+	+	+	-	-	-	-	d	d
Gluconate dehydrogenase	+	+	-	-	-	-	-	-	d
$\beta$ -Xylosidase test	+	-	+	+	-	+	+	+	d
Lysine decarboxylase test	+	+	-	-	-	-	-	-	-
Arginine "dihydrolase" test	-	-	+	-	+	+	+	+	-
Ornithine decarboxylase test	+	+	+	+	+	+	+	+	-
Urea hydrolyzed	-	+	-	-	-	-	-	-	-
Esculin (black color)	+	+	+	+	+	+	+	d	v
Acid production from:									
Adonitol	+	-	-	-	-	-	-	v	v
D-Arabitol	+	+	-	-	-	-	-	v	v
Dulcitol	-	-	-	+	-	-	-	d	d
$\alpha$ -Methyl-D-glucoside	+	-	+	+	v	v	+	+	v
Raffinose	+	+	+	+	-	+	v	+	v
D-Sorbitol	+	-	-	+	-	-	+	+	v
Sucrose	+	+	+	v	-	+	-	+	v



Table 2. *Continued*

Test	<i>E. aerogenes</i>	<i>E. gergoviae</i>	<i>E. sakazakii</i>	<i>E. intermedium</i>	<i>E. cancerogenus</i>	<i>E. amnigenus</i>	<i>E. nimipressuralis</i>	<i>E. cloacae</i> complex	<i>E. agglomerans</i> complex
Utilization of:									
<i>cis</i> -Aconitate	+	v	+	-	+	-	-	v	d
<i>trans</i> -Aconitate	+	-	v	-	+	-	-	v	d
Adonitol	+	-	-	-	-	-	-	d	d
4-Aminobutyrate	v	+	+	-	-	-	-	-	v
5-Aminovalerate	v	+	-	-	-	-	-	-	-
D-Arabitol	+	+	-	-	-	-	-	d	d
L-Arabitol	-	-	-	-	-	-	-	-	d
Benzoate	+	v	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	d
<i>m</i> -Coumarate	-	+	-	-	-	-	-	-	-
Dulcitol	v	-	v	+	-	-	-	d	d
<i>i</i> -Erythritol	-	-	-	-	-	-	-	-	d
L-Fucose	+	v	-	v	+	-	-	d	v
Gentisate	+	v	-	-	-	-	-	-	-
Histamine	(+)	+	-	-	-	-	-	-	-
3-Hydroxybenzoate	+	v	-	-	-	-	-	-	-
4-Hydroxybenzoate	v	+	-	+	-	-	-	-	v
3-Hydroxybutyrate	(+)	v	-	-	-	-	-	v	-
<i>myo</i> -Inositol	+	v	v	-	-	-	-	d	d
5-Ketogluconate	v	+	-	+	-	-	-	-	d
2-Ketoglutarate	v	v	-	(v)	v	-	-	v	v
Lactose	+	(+)	+	+	(v)	+	+	(+)	d
Lactulose	(+)	v	+	v	-	v	-	v	d
D-Lyxose	-	-	-	-	-	+	+	d	d
D-Malate	+	v	(v)	-	v	-	v	v	v
L-Malate	+	+	+	v	+	+	+	+	v
Malonate	v	v	(v)	-	(v)	-	-	v	-
Maltitol	+	-	+	+	-	+	+	+	-
D-Melibiose	+	+	+	+	-	+	+	d	d
1- <i>O</i> -Methyl- $\alpha$ -galactoside	+	+	+	+	-	+	+	d	d
3- <i>O</i> -Methyl-D-glucose	-	-	-	-	+	-	-	d	-
1- <i>O</i> -Methyl- $\alpha$ -D-glucoside	+	-	+	+	-	v	+	+	-
Mucate	+	-	-	+	+	+	+	v	d
Palatinose	+	-	+	+	-	+	+	+	-
Phenylacetate	+	+	-	v	+	-	+	d	d
L-Proline	+	+	+	-	+	v	+	+	+
Protocatechuate	+	+	-	+	-	-	-	-	d
Putrescine	v	v	+	-	+	-	-	d	-
Quinate	+	+	-	+	-	-	-	-	d
D-Raffinose	+	+	+	+	-	+	v	v	d
L-Rhamnose	+	+	+	+	+	+	+	d	+
D-Saccharate	+	-	-	+	+	+	+	+	d
D-Sorbitol	+	-	-	+	-	-	+	+	d
Sucrose	+	+	+	v	-	+	-	+	d
D-Tagatose	v	-	-	+	-	-	v	v	-
D-Tartrate	-	-	-	-	-	-	-	-	d
L-Tartrate	v	-	-	-	-	-	-	-	v
<i>meso</i> -Tartrate	(v)	v	-	-	-	-	-	-	v
Tricarallylate	+	v	-	-	-	-	-	-	-
Trigonelline	v	-	-	-	-	-	-	-	d
Tryptamine	v	-	-	-	-	-	-	-	-
D-Turanose	v	-	v	v	-	(v)	v	v	-
L-Tyrosine	v	v	-	-	-	-	-	v	-
Xylitol	v	-	-	-	-	-	-	-	d
D-Xylose	+	+	+	+	+	+	+	+	d

Symbols: +, 95–100% strains positive in 1–2 days; (+), 95–100% strains positive in 1–4 days; -, 95–100% strains negative in 4 days; v, positive or negative in 1–4 days; d test used to differentiate species within a complex.

Table 3. Phenotypic properties of genomic groups within the *Enterobacter cloacae* complex.

Test	Genomic group or subgroup <sup>a</sup>						
	1	2	3	4a	4b	4c	5
Glucose dehydrogenase	–	–	+	–	–	–	–
Motility	+	+	+	–	v	+	+
Malonate test	+	+	+	–	–	v	+
Esculin hydrolyzed	v	–/(+)	–/(+)	+	+	+	+
Utilization of:							
Adonitol	–	–	v	–	–	–	–
D-Arabitol	–	–	v	–	–	–	–
Dulcitol	–	v	v	–	–	v	+
Fucose	–	–	v	–	–	–	–
D-Galacturonate	+	v	+	+	+	+	+
<i>myo</i> -Inositol	+	+	v	+	+	+	+
Lyxose	v	–	+	+	+	v	+
D-Melibiose	+	+	v	–	+	+	+
3-Methylglucose	–	–	v	–	–	–	–
Phenylacetate	v	+	+	–	+	+	+
Putrescine	v	+	–	+	–	+	–
D-Raffinose	+	+	v	v	v	+	+
L-Rhamnose	+	+	+	–	v	–	+
Sorbitol	+	+	v	+	+	+	+
Xylitol	–	–	(v)	–	–	–	–

Symbols: +, 95–100% strains positive in 1–2 days (utilization tests) or in 1 day (other tests); (+), 95–100% strains positive in 1–4 days; –, 95–100% strains negative in 4 days; v, positive or negative in 1–4 days.

<sup>a</sup>The type strain of *E. dissolvens* and the present type strain of *E. cloacae* are in genomic group 1; the type strain of *E. hormaechei* is in genomic group 3; and the type strain of *E. asburiae* is in genomic group 4, subgroup 4a.

Table 4. Observed biogroups in genomic group 3 (in the *E. cloacae* complex).

Test	Biogroup						
	3a <sup>a</sup>	3b	3c	3d	3e	3f	3g
Utilization of:							
D-Melibiose	–	+	+	+	–	+	+
D-Raffinose	–	+	+	+	+	+	+
Methyl- $\alpha$ -D-galactoside	–	+	+	+	–	+	+
Fucose	v	+	+	–	+	+	+
D-Arabitol	–	–	–	–	–	+	+
Sorbitol	–	+	+	+	+	+	+
Adonitol	–	–	–	–	–	+	+
3-Methylglucose	+	–	+	–	–	+	–

Symbols: +, all strains positive in 1–2 days (carbon source utilization tests) or in one day (other tests); (+), all strains positive in 1–4 days; –, all strains negative in 4 days; v, positive or negative in 1–4 days.

<sup>a</sup>Five strains representing *E. hormaechei* (including the type strain) and received from the Centers for Disease Control corresponded to biogroup 3a.

from genomic group 2. Genomic group 3 (containing the type strain of *E. hormaechei*) is unique in oxidizing D-glucose to D-gluconate without added pyrroloquinoline quinone. Phenotypic identification of genomic group 4 is easier at the subgroup level. Subgroups 4a (containing the type strain of *E. asburiae*), 4b, and 4c are differentiated by utilization of dulcitol, D-melibiose, phenylacetate, putrescine, and L-rhamnose. Genomic group 5 utilizes dulcitol, phenylacetate, and L-rhamnose, and does not utilize putrescine. Genomic groups 4 and 5 hydrolyze esculin rapidly.

The diversity of biogroups within *E. cloacae* genomic group 3 is shown in Table 4. Strains presently identified as *E. hormaechei* belong in biogroup 3a. These strains are negative for utilization of D-melibiose, D-raffinose,  $\alpha$ -methyl-D-galactoside, sorbitol, and adonitol. Most clinical isolates recovered from blood and received as *E. cloacae* from hospitals in Paris corresponded to genomic group 3, biogroups 3b, 3d and 3f.

*Enterobacter* strains that are negative in lysine and ornithine decarboxylases and arginine dihydrolase, whether or not they produce a yellow pigment, are currently identified as *E. agglomer-*

Table 5. Differential phenotypic characteristics of genomic species (I to XIII) within the *Enterobacter agglomerans* complex.

Test	Pantoea						Leclercia		Other groups			
	XIII	V	III	I	II	IV	VI	XI	VII	VIII	IX	XII
Glucose dehydrogenase	+	+	+	+	+	+	+	-	-	v	+	-
Gluconate dehydrogenase	+	+	+	+	+	+	+	-	-	v	-	-
Gas from glucose	-	-	-	-	-	-	-	+	-	-	+	+
Indole	-	-	-	-	-	-	+	+	+	-	-	+
Malonate (Leifson)	+	+	-	-	+	-	-	+	+	+	-	+
$\beta$ -Xylosidase	+	+	-	+	-	-	+	v	+	-	+	+
Utilization of:												
Adonitol	(v)	-	-	-	-	+	-	+	v	(v)	-	v
D-Arabitol	v	v	+	-	v	+	+	+	v	-	-	+
L-Arabitol	-	-	-	-	-	+	-	-	-	-	-	-
Dulcitol	-	v	(v)	-	-	-	-	+	-	-	+	v
meso-Erythritol	-	-	+	-	-	v	-	-	-	v	-	-
Gentiobiose	-	v	+	+	-	v	+	+	+	v	+	+
myo-Inositol	+	+	+	+	v	+	v	-	-	v	-	-
5-Ketogluconate	-	(v)	+	+	v	+	(+)	-	-	+	-	v
Lactose	-	v	-	+	v	-	+	+	+	-	v	(+)
Melibiose	-	-	-	-	-	-	+	+	+	v	+	-
Phenylacetate	-	-	-	-	-	-	-	+	+	-	-	-
Protocatechuate	-	-	-	-	-	-	v	-	-	-	-	-
Quinate	-	-	-	-	-	-	v	-	-	(v)	-	-
D-Sorbitol	v	-	-	+	(v)	-	v	-	-	-	+	v
Sucrose	v	v	+	-	v	+	+	v	v	v	+	+
D-Tartrate	+	-	-	-	-	-	-	-	-	-	-	-
L-Tartrate	-	v	v	-	v	-	-	-	-	-	-	v
meso-Tartrate	(v)	(v)	v	-	+	-	v	-	-	-	-	v
Trigonelline	v	-	-	-	v	+	-	-	-	-	-	-
Xylitol	-	-	-	-	v	+	-	(v)	-	+	-	-
D-Xylose	+	+	+	+	+	-	+	+	+	-	+	+

Symbols: +, 95–100% strains positive in 1–2 days (utilization tests) or in 1 day (other tests); (+), 95–100% strains positive in 1–4 days; -, 95–100% strains negative in 4 days; v, positive or negative in 1–4 days.

*ans*. Several biotypes were described (Ewing and Fife, 1972). The differential phenotypic properties of strains belonging to the major genomic groups of Brenner et al. (1984) are shown in Table 5 (P.A.D. Grimont, E. Ageron, and D.J. Brenner, unpublished observations). DNA groups XIII, III, VI, and XI correspond respectively to *Pantoea agglomerans*, *P. dispersa*, “*Erwinia*” *ananas*, and *Leclercia adecarboxylata*. Anaerogenic groups XIII, V, III, I, II, IV, and VI are able to oxidize D-glucose to D-gluconate (glucose dehydrogenase activity) without added pyrroloquinoline quinone, and D-gluconate to 2-ketogluconate (gluconate dehydrogenase activity). In addition, these groups cannot utilize phenylacetate, and most strains can utilize *myo*-inositol. These tests could define the genus *Pantoea*.

Genomic groups XI and VII are very similar (indol produced, malonate test positive, utilization of phenylacetate) although only group XI (*Leclercia adecarboxylata*) is aerogenic.

Table 5 is oversimplified since a number of strains that did not fall into DNA groups I to XIII (Brenner et al., 1984) are not included.

### Serotyping of *Enterobacter cloacae*

Sakazaki and Namioka (1960) distinguished 53 O and 56 H antigens in agglutination tests, and 170 isolates were distributed in 79 serotypes. Bacterial suspensions had to be boiled to be agglutinated by O antisera. Unfortunately, epidemiological investigations using this serotyping scheme have not been reported.

Gaston et al. (1983) devised a serotyping scheme based on heat-stable somatic (O) antigens. A total of 28 antisera including 11 absorbed sera were used, thus defining 28 O serogroups. Of 300 clinical isolates from 66 hospitals, 78% were typable, 11% were not agglutinated by any of the sera, and 11% were autoagglutinable in saline.

### Capsular Typing of *Enterobacter aerogenes*

About 81% of *E. aerogenes* strains are surrounded by a thin capsule antigenically related to *Klebsiella* capsular antigens. Thus, *Klebsiella* K antisera can be used to type strains of *E. aero-*

*genes*. The following antisera reacted with *E. aerogenes* capsular antigens: K4, K4+59, K11, K26, K42, K59, and K68 (Richard, 1977). Strains of *E. aerogenes* are not agglutinated by *E. cloacae* O antisera (Gaston et al., 1989).

### Serotyping of *Enterobacter agglomerans*

The principal antigens in most strains of *E. agglomerans* (*Erwinia herbicola*) are uncharged capsular polysaccharides. These antigens are not removed from the cells by heating at 100°C for 30 min (Slade and Tiffin, 1984). Most members of the "herbicola group" are motile, but there is no report on the antigenic structure of the flagella (Slade and Tiffin, 1984), and most strains produce antigenically similar, high-molecular-weight, acidic polysaccharides.

Two schemes have been proposed for "*E. herbicola*." Muraschi et al. (1965) used immunodiffusion and antigens extracted by aqueous ether. They classified 55 isolates into seven serotypes, although "some cultures were mixtures of more than one serotype." A second scheme was established by Slade (cited by Slade and Tiffin, 1984), using laboratory strains. This scheme has not been used to type new isolates.

### Bacteriocin Typing of *Enterobacter cloacae*

Three different schemes have been developed for typing *E. cloacae* strains by susceptibility to bacteriocins:

1. Freitag and Friedrich (1981) were able to type 51 of 65 strains (78%), which were assigned to 23 bacteriocin types designated A through X (type Y contained nontypable isolates).

2. Traub et al. (1982) found 9% of 256 *E. cloacae* isolates to be bacteriocinogenic. Bacteriocins were produced by 16 strains after induction by mitomycin. A total of 308 isolates from various clinical sources were studied and 79% fell into 52 bacteriocin types leaving 21.4% of the isolates untypable.

3. Reference is not an exact match Bauernfeind and Petermüller (1984) found 132 of 149 isolates to produce bacteriocins. With a set of eight bacteriocin-producing strains, typability of 134 clinical isolates was 96.3%. A total of 44 different bacteriocin types could be distinguished. Only 11 (8.2%) of the isolates fell into the largest bacteriocin type. Other species of *Enterobacter* (*E. agglomerans* and *E. aerogenes*) were also typable by the selected *E. cloacae* bacteriocins.

### Phage Typing of *Enterobacter cloacae*

From sewage Gaston (1987a) isolated 76 phages active against *E. cloacae*. Of these, 26 phages

were selected after numerical taxonomy analysis of their reaction patterns on 92 *E. cloacae* strains. This system has been tried on 384 isolates and 94% of these isolates were susceptible to at least one phage. A total of 325 different phage susceptibility patterns were observed. Reproducibility of patterns was 100% when duplicate testing was on the same day, and only 40% when duplicate testing was done after 18 months (Gaston, 1987b).

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# The Genus *Hafnia*

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## Introduction

The genus *Hafnia* was recognized by Møller (1954) while studying amino acid decarboxylase patterns among Enterobacteriaceae. Members of this taxon are Gram-negative, facultatively anaerobic rods that are typically methyl red and Voges-Proskauer positive, positive for lysine and ornithine decarboxylase, and negative for arginine dihydrolase. The taxonomic position of these organisms has been the subject of some controversy, and over the years they have been referred to as *Bacillus asiaticus* (cited by Ewing and Fife, 1968), *Bacterium cadaveria* (Gale and Epps, 1943), *Aerobacter* species biotype 32011 (Stuart et al., 1943b), *Enterobacter alvei* (Sakazaki, 1961), *Enterobacter aerogenes* subsp. *hafniae* (Ewing, 1963), and *Enterobacter hafniae* (Ewing and Fife, 1968). However, DNA hybridization studies have shown that these organisms are distinct from *Enterobacter* (Steigerwalt et al., 1976), and *Hafnia alvei* (the only recognized species of the genus) is a legitimate taxon.

Barbe described two *H. alvei* biotypes, which can be distinguished by hydrolysis of arbutin and esculin and fermentation of salicin and D-arabinose (cited in Introduction to the Family Enterobacteriaceae in the second edition). Janda et al. (2002) subsequently identified isolates that fell into two additional biogroups (Table 1). These authors reported that isolates belonging to biogroup 2 were less common than isolates belonging to biogroup 1 and that isolates belonging to biogroups 3 and 4 were rare (Janda et al., 2002). Both DNA relatedness (Steigerwalt et al., 1976) and 16S ribosomal RNA gene sequencing (Janda et al., 2002) indicate that there are at least two distinct genomospecies of *H. alvei*. Because the genomospecies cannot be clearly distinguished by phenotypic characteristics, they remain classified as a single heterogeneous species. It has been suggested that genomospecies 1, which includes the majority of isolates as well as the type strain (ATCC 13337), should be considered *H. alvei* sensu stricto (Janda et al., 2002).

## Habitat

*Hafnia alvei* can be isolated from feces in apparently healthy individuals, as well as from mammals, birds, reptiles and fish. It can also be isolated from a range of environmental sources, including soil, water and sewage, as well as from food. Early studies incriminated biotype 32011 of paracolon bacteria, now classified as *H. alvei*, as a cause of diarrhea (cited in The Genus *Hafnia* in the second edition). Diarrhea-associated isolates, more recently identified as *H. alvei* and shown to possess a locus of enterocyte effacement (LEE) pathogenicity island and to produce attaching and effacing lesions (Albert et al., 1991; Albert et al., 1992), were subsequently found to be distinct from true *H. alvei* (Ridell et al., 1995; Ismaili et al., 1996; Janda et al., 1999). These strains are now classified as *Escherichia albertii* (Abbott et al., 2003; Huys et al., 2003). Thus, the role of *H. alvei* as a primary cause of gastroenteritis and diarrheal disease remains uncertain.

The main clinical significance of *H. alvei* is as a rare cause of opportunistic infections, particularly in compromised hosts. *Hafnia alvei* has been isolated from urine, blood, wound and abscess exudate, and sputum, in addition to stool specimens. *Hafnia alvei* has been implicated as a cause of septicemia, endocarditis, meningitis, peritonitis, pneumonia, urinary tract infections, liver abscesses, and cholangitis (reviewed by Englund [1969], Conte et al. [1996], Günthard and Pennekamp [1996], Barry et al. [1997], and Ramos and Damaso [2000]). Because *H. alvei* is often found in mixed cultures, it can be difficult to ascribe unequivocal clinical significance to the organism. *Hafnia alvei* causes both nosocomial and community acquired infections. The vast majority of patients with *H. alvei* infections have underlying illnesses.

## Isolation

As reviewed in The Genus *Hafnia* in the second edition, there are no specific media for the isola-

Table 1. Biotypes of *Hafnia alvei*.

Biotype	Biochemical reactions <sup>a</sup>			
	Esculin	Arbutin	Salacin	D-Arabinose
1	–	–	–	+
2	+	+	+	–
3	+	+	+	+
4	–	–	–	–

Symbols: +, present; and –, absent.

<sup>a</sup>Esculin and arbutin hydrolysis at 48h, salacin and D-arabinose fermentation at 72h. Modified from Janda et al. (2002).

tion of *H. alvei*, but media commonly employed for isolating other Enterobacteriaceae can be used. In the absence of a specific enrichment broth, selenite and tetrathionate are suitable for the recovery of some *H. alvei* strains, but other strains will not grow in these media. Some strains will grow at 4°C, and care must be taken to distinguish these isolates from *Yersinia* species if cold enrichment is used. Plating media, such as eosin methylene blue (EMB), MacConkey lactose, xylose-lysine-deoxycholate (XLD), and Hektoen, have been used to isolate *H. alvei*. Because it does not ferment lactose, *H. alvei* forms colorless colonies on these media, and care should be taken to distinguish it from *Salmonella* species. Highly selective media, such as *Salmonella-Shigella* (SS), can inhibit the growth of up to 25% of *H. alvei* strains (The Genus *Hafnia* in the second edition). Growth on less inhibitory media yields smooth, translucent colonies with complete edges. *Hafnia alvei* rarely produces mucoid colonies. Sakazaki recommended the use of MacConkey agar containing 1% sorbitol (SMAC) to isolate *H. alvei* (cited in The Genus *Hafnia* in the second edition). Because it does not ferment sorbitol, its colorless colonies can be distinguished from Enterobacteriaceae that do ferment this sugar, including many strains of *E. coli*, *Citrobacter*, *Klebsiella*, *Enterobacter* and *Serratia*. Enterohemorrhagic *E. coli* O157:H7 does not ferment sorbitol and is often isolated on SMAC, therefore fecal isolates of *H. alvei* must be distinguished from *E. coli* O157:H7.

## Identification

Conforming to the definition of the family Enterobacteriaceae, members of the genus *Hafnia* are Gram-negative, facultatively anaerobic rods, and typically motile by peritrichous flagella (see Introduction to the Family Enterobacteriaceae in the second edition). Isolates of *H. alvei* are typically not encapsulated. Identification is most commonly based on patterns of biochemical reactions (Table 2). Sakazaki and Tamura (The Genus *Hafnia* in the second edition) indicate

Table 2. Biochemical reactions of *Hafnia alvei*.

Test <sup>a</sup>	Reaction
Indole production	–
Methyl red	d
Voges-Proskauer	[+]
Citrate (Simmons)	–
H <sub>2</sub> S (triple sugar iron)	–
Urease	–
Phenylalanine deaminase	–
Lysine decarboxylase	+
Arginine dihydrolase	–
Ornithine decarboxylase	+
Gelatin hydrolysis	–
Malonate utilization	d
Tartrate (Jordan's)	d
Esculin hydrolysis	–
DNase	–
Lipase	–
ONPG	+
Acid produced from	
D-Adonitol	–
L-Arabinose	+
Cellobiose	[–]
Dulcitol	–
Glycerol	+
Inositol	–
Lactose	–
Maltose	+
D-Mannitol	+
Melibiose	–
Raffinose	–
L-Rhamnose	+
Salicin	[–]
D-Sorbitol	–
Sucrose	–
Trehalose	+
D-Xylose	+
Mucate	–

Symbols and abbreviation: –, ≤10% positive; [–], 11–25% positive; d, 26–75% positive; [+], 76–89% positive; +, ≥90% positive; and ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

<sup>a</sup>Assays performed at 36 ± 1°C, except gelatin hydrolysis (22°C) and DNase (25°C).

From Holt et al. (1994).

that the variable methyl red and Voges-Proskauer reactions seen with *H. alvei* at 35°C are consistently negative and positive, respectively, when carried out at 22–25°C. Likewise, citrate utilization on Simmons' citrate media is negative at 35°C but often delayed positive (3–4 days) at 22–25°C. These authors also report that motility is also increased when cultures are incubated at room temperature.

In the clinical laboratory, *H. alvei* must be differentiated from members of the closely related genera *Enterobacter* and *Serratia*. This can be accomplished with selected biochemical tests (Table 3). *Hafnia alvei* is negative (or delayed positive) for citrate utilization, while *Enterobacter* and *Serratia* are positive. *Hafnia alvei* is

Table 3. Differential characteristics of the genus *Hafnia* and biochemically similar genera.

Test <sup>a</sup>	Biochemical reactions <sup>b</sup>		
	<i>H. alvei</i>	<i>Enterobacter</i>	<i>Serratia</i>
Citrate (Simmons')	–	+	+
Lysine decarboxylase	+	–	D
Arginine dihydrolase	–	D	–
Gelatin hydrolysis	–	–	+
DNase	–	–	+
Lipase (Tween 80)	–	–	+
Acid produced from			
D-Adonitol, lactose, inositol	–	D	D
Raffinose	–	D	D
Sucrose	–	+	+

Symbols: –, ≤10% positive; [–], 11–25% positive; d, 26–75% positive; [+], 76–89% positive; +, ≥90% positive; D, different reactions given by different species of a genus.

<sup>a</sup>Assays performed at 36 ± 1°C, except gelatin hydrolysis (22°C) and DNase (25°C).

<sup>b</sup>*Enterobacter* exceptions are for lysine decarboxylase (*E. gergoviae*), gelatin hydrolysis (*E. nimipressuralis*), and sucrose fermentation (*E. amnigenus* biogroup 2, *E. nimipressuralis*, and *E. taylora*). *Serratia* exceptions are for arginine dihydrolase (*S. grimesii*), gelatin hydrolysis and DNase (*S. fonticola*), lipase (*S. entomophila* and *S. fonticola*), and sucrose fermentation (*S. fonticola* and *S. odorifera* biogroup 2).

From Holt et al. (1994).

lysine decarboxylase positive, while *Enterobacter* species, with the exception of *Enterobacter gergoviae*, are negative. *Hafnia alvei* is negative for DNase activity, gelatin hydrolysis, and lipase activity, while *Serratia* species are typically positive for these tests, with the exception of *Serratia fonticola*. Some strains of *Serratia entomophila* are also negative for lipase.

*Escherichia albertii* in Bangladeshi children with diarrhea was initially misidentified as *H. alvei* (Albert et al., 1991). Abbott et al. (2003) found that 4 of 5 *E. albertii* strains were identified as *H. alvei* using API 20E strips (bioMérieux, Inc.), albeit at low probabilities, and 3 of 5 of these strains were identified as *H. alvei* using Vitek GNI Plus (bioMérieux, Inc.). The weak to moderate L-proline aminopeptidase activity noted in *E. albertii* strains is a useful test to distinguish them from *H. alvei*. *Hafnia alvei* exhibit strong L-proline aminopeptidase activity within 30 min to 2 h (Abbott et al., 2003).

Perhaps the most reliable method for identification of *H. alvei* is the *Hafnia*-specific bacteriophage 1672 described by Guinée and Valkenburg (1968). All *H. alvei* strains are lysed by the phage but not strains of *Enterobacter*, *Klebsiella*, *Citrobacter*, *Serratia* or *Salmonella* (cited by in The Genus *Hafnia* in the second edition). *Escherichia albertii* strains are also not lysed by *Hafnia* phage 1672 (Janda et al., 1999).

Identification of *H. alvei* with Phage 1672 (Guinée and Valkenburg, 1968).

The specific phage 1672 was isolated from surface water with *H. alvei* strain 1672 as the propagating bacterium.

Surface inoculate a well-dried nutrient agar plate with a fresh broth culture of the strain to be tested. After decantation, allow the plate to dry at room temperature for 15 min. Spot a drop of the undiluted phage 1672 on the plate with a Pasteur pipette, and allow the plate to dry again. Read after 16–20 h of incubation at 35°C. Clear plaques with a diameter of 1–2 mm are produced. Obtain the phage preparation (ca. 10<sup>9</sup> plaque-forming units per ml) after the usual purification. The phage is not inactivated by heating at 60°C for 30 min.

Stuart and Rustigian (1943a) characterized biotype 32011 of paracol bacteria, now classified as *H. alvei*, dividing it into 8 serovars. Subsequent studies identified additional O and H serogroups (Deacon, 1952; Eveland and Faber, 1953), but some of these organisms are now recognized to be species other than *H. alvei* (cited in The Genus *Hafnia* in the second edition). More recently, chemical studies have elucidated the structural nature of the lipopolysaccharide (LPS) and O antigen of several *H. alvei* strains (reviewed by Romanowska, 2000). Although serotyping currently has little practical application for identification of *H. alvei*, it should be noted that some isolates cross-react with O antisera of *Salmonella* species and *E. coli* (The Genus *Hafnia* in the second edition).

## Antibiotic Resistance

The majority of *H. alvei* strains are sensitive to carbenicillin, streptomycin, gentamycin, kanamycin, chloramphenicol, tetracycline, polymyxin B and nalidixic acid, but resistant to ampicillin and cephalothin (The Genus *Hafnia* in the sec-

ond edition). Günthard and Pennekamp (1996) found *H. alvei* isolates from patients with community acquired and nosocomial infections were all sensitive to ciprofloxacin and imipenem. Approximately 90% of the isolates were also sensitive to the third generation cephalosporins (ceftriaxone and ceftazidime). With a more limited number of isolates, Ramos and Damaso (2000) reported some resistance to amoxicillin/clavulanic acid and first generation cephalosporins but also found that all isolates were sensitive to aminoglycosides, ciprofloxacin, imipenem, and the third generation cephalosporin cefotaxime.

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## The Genus *Serratia*

FRANCINE GRIMONT AND PATRICK A. D. GRIMONT

The genus *Serratia* a member of the Enterobacteriaceae (see Introduction to the Family Enterobacteriaceae from the second edition.), is comprised of a group of bacteria that are related both phenotypically and by DNA sequence. The type species of the genus is *Serratia marcescens*. Some species and biotypes of *Serratia* produce a nondiffusible red pigment, prodigiosin, or 2-methyl-3-amyl-6-methoxyprodigiosene (Williams and Qadri, 1980). The multiplication of red-pigmented *Serratia* was incriminated in the appearance of bloodlike spots (e.g., on bread, consecrated wafers [sacramental Hosts], and polenta) with rather disastrous sociological consequences. In this context, several scholars have traced the history of the genus *Serratia* back to antiquity (Gaughran, 1969; Harrison, 1924; Reid, 1936). However, several bacterial species outside the genus *Serratia* produce prodigiosin or prodigiosin-like pigments (Williams and Qadri, 1980) or many other kinds of red pigments, and the identity of microorganisms involved in these striking phenomena can only be surmised.

Bizio (1823) named the red-pigmented microorganism he observed on polenta *Serratia marcescens*. Ehrenberg (1848) named a motile bacterium isolated from red spots on food “*Monas prodigiosa*.” No cultures of these organisms were preserved, but the name *Serratia marcescens* was preferred over the name “*Erythrobacillus pyosepticus*”—a culture of which was preserved as ATCC 275 (Fortineau, 1904)—by Breed and Breed (1924, 1927) and by the editors of *Bergey’s Manual of Determinative Bacteriology* (Bergey et al., 1923). The name *Serratia marcescens* is now universally accepted, and a neotype strain has been designated (Martinec and Kocur, 1961a).

At the start of this century, more than 76 nomenclatures had been described with red or pink pigmentation (Hefferan, 1904), and 23 *Serratia* species were listed in the first edition of *Bergey’s Manual* (Bergey et al., 1923). This number progressively decreased to five in the fifth edition of *Bergey’s Manual* (Breed et al., 1957), and later to one species: *S. marcescens* (Ewing et al., 1959; Martinec and Kocur, 1960, 1961a, 1961b, 1961c, 1961d). The only *Serratia* species recognized in the eighth edition of *Bergey’s Manual* was *S. marcescens* (Sakazaki, 1974). Then, new objective approaches, such as numerical taxonomy and DNA relatedness applied to strains recovered from diverse habitats, delineated an increasing number of species in the genus *Serratia*. Five and seven species, respectively, were mentioned in the first edition of *The Prokaryotes* (Grimont and Grimont, 1981) and in *Bergey’s Manual of Systematic Bacteriology* (Grimont and Grimont, 1984). Ten species are presently known to belong in the genus *Serratia*. These species (and synonyms) are:

1. *Serratia marcescens* Bizio 1823: “Typical” *S. marcescens* (Colwell and Mandel, 1965; Ewing et al., 1959; Martinec and

Kocur, 1961a); *Serratia* pattern 1 (Fulton et al., 1959); *Serratia* biotype 1 (Bascomb et al., 1971); phenon A (Grimont and Dulong de Rosnay, 1972; Grimont et al., 1977b); *S. marcescens* DNA hybridization group (Steigerwalt et al., 1976). The neotype strain is ATCC 13880, CDC 813-60, Grimont 504, and CCM 303 (Martinec and Kocur, 1961a). The name appeared in the *Approved Lists of Bacterial Names* (Skerman et al., 1980). The type strains of “*Bacillus indicus*” (Eisenberg, 1886), “*Erythrobacillus pyosepticus*” (Fortineau, 1904), “*Bacillus sphingidis*” (White, 1923a), and “*Serratia anolium*” (Duran-Reynals and Clausen, 1937) are all referable to the taxonomic entity now known as *S. marcescens*. Strains labeled “*S. marcescens* subsp. *kiliensis*” according to Ewing et al. 1962 were Voges-Proskauer-negative variants of *S. marcescens*.

2. *Serratia liquefaciens* (Grimes and Hennerty 1931) Bascomb et al. 1971: “*Aerobacter liquefaciens*” Grimes and Hennerty 1931; “*Aerobacter lipolyticus*” Grimes 1961; *Enterobacter liquefaciens* (Grimes and Hennerty, 1931) Ewing 1963; phenon Clab (Grimont et al., 1977b); *Serratia liquefaciens* sensu stricto (Grimont et al., 1982a, 1982b). The type strain is ATCC 27592, CDC 1284-57, and Grimont 866. The name appeared in the *Approved Lists of Bacterial Names* (Skerman et al., 1980). It should be mentioned that the strain considered to be the type strain of “*Aerobacter liquefaciens*,” or of “*Aerobacter lipolyticus*” by Grimes (1961), was ATCC 14460 (now the type strain of *S. grimesii*). Since this strain was considered atypical, another strain (ATCC 27592) was given as the type strain in the *Approved Lists*.

3. *Serratia proteamaculans* (Paine and Stansfield 1919) Grimont et al. 1978b: *S. proteamaculans* sensu stricto (Grimont et al., 1982a, 1982b). The type strain is ATCC 19323, Grimont 3630, ICPB XP176, and NCPPB 245. The name appeared in the *Approved Lists of Bacterial Names* (Skerman et al., 1980). *S. proteamaculans* and *S. liquefaciens* were thought to be synonymous on the basis of DNA relatedness (Grimont et al., 1978b). However, subsequent observation of significant thermal instability of DNA hybrid fragments supported the separation of both species (Grimont et al., 1982a).

Biogroup RQ was named *S. proteamaculans* subspecies *quinovora* (Grimont et al., 1982a, 1982b) with strain Grimont 4364, CIP 8195, and ATCC 33765 as the type strain.

4. *Serratia grimesii* Grimont et al. 1982a, 1982b: phenon Cld (Grimont et al., 1977b); *S. liquefaciens* hybridization group (Steigerwalt et al., 1976). The type strain is ATCC 14460 and Grimont 503. This strain had been considered as the type strain of *S. liquefaciens* prior to the publication of the *Approved Lists*.

5. *Serratia plymuthica* (Lehmann and Neumann 1896) Breed et al. 1948: “*Bacterium plymuthicum*” Lehmann and

Neumann 1896; excluded from the genus *Serratia* (Ewing et al., 1959); “atypical” *S. marcescens* (Colwell and Mandel, 1965); “*S. marcescens* var. *kiliensis*” according to Martinec and Kocur (1961d) (not Ewing et al., 1962); *Serratia* III (Mandel and Rownd, 1964); *Serratia* pattern 2 (Fulton et al., 1959); atypical *S. rubidaea* (Ewing et al., 1972, 1973); and phenon C2 (Grimont et al., 1977b). The type strain is Grimont 510, CCM 640, and ATCC 183. The type strains of “*Bacterium kiliense*” reference is not an exact match Lehmann and Neumann 1986 and “*Serratia esseyana*” Combe 1933 are *S. plymuthica*.

6. *Serratia rubidaea* Stapp 1940 (Ewing et al., 1973): “*Bacterium rubidaeum*” Stapp 1940; “*Prodigiosus*” VIII (Hefferan 1904); *S. marinorubra* ZoBell and Upham 1944; *Serratia* biotype 2 (Bascomb et al., 1971); phenon B (Grimont and Dulong de Rosnay, 1972; Grimont et al., 1977b); *S. rubidaea* DNA hybridization group (Steigerwalt et al., 1976). The name appeared in the *Approved Lists of Bacterial Names* (Skerman et al., 1980). The type strain (neotype) is ATCC 27593, CDC 2199-72, and Grimont 864. The former type strain (holotype) of *S. marinorubra* was NCTC 10912, ATCC 27614, and Grimont 288. However, the *Approved Lists of Bacterial Names* (Skerman et al., 1980) listed *S. marinorubra* with ATCC 27593 as the type strain. Thus, both names (*S. rubidaea* and *S. marinorubra*) were made objective synonyms and the name *S. rubidaea* has priority. Three subspecies can be delineated: *S. rubidaea* subsp. *rubidaea*, *S. rubidaea* subsp. *burdigalensis*, and *S. rubidaea* subsp. *colindalensis* (Grimont et al., manuscript in preparation).

7. *Serratia odorifera* Grimont et al. 1978a: Strains similar to the unclustered *Serratia* strain 38 (Grimont and Dulong de Rosnay, 1972; Grimont et al., 1977b). The type strain is ATCC 33077, CDC 1979-77, Grimont 1073, ICPB 3995, and NCTC 11214. The name appeared in the *Approved Lists of Bacterial Names* (Skerman et al., 1980).

8. *Serratia ficaria* Grimont et al. 1979c: the type strain is ATCC 33105, Grimont 4024, CIP 79.23, and ICPB 4050.

9. *Serratia entomophila* Grimont et al. 1988: the type strain is ATCC 43705, Jackson A1, and CIP 102919.

10. *Serratia fonticola* Gavini et al. 1979: Lysine-positive *Citrobacter*-like (Steigerwalt et al., 1976). The type strain is ATCC 29844, CUETM 77.165, Grimont 4011, and CIP 78.64. The name appeared in the *Approved Lists of Bacterial Names* (Skerman et al., 1980).

The inclusion of the genus *Serratia* in the tribe Klebsiellae is no longer tenable. Studies on DNA relatedness, immunological cross-reaction between isofunctional enzymes, and the physical properties, regulation, and amino acid sequences of enzymes have all shown the genus *Serratia* to be consistently different from the group composed of the genera *Escherichia*, *Shigella*, *Salmonella*, *Citrobacter*, *Klebsiella*, and *Enterobacter* (reviewed by Grimont and Grimont, 1978a).

Until the late 1950s, *Serratia* spp. were rarely isolated from human patients. Later on *S. marcescens* became more and more frequently involved in nosocomial infections and non-pigmented *S. marcescens* strains are now a serious threat in surgical and intensive care units (Daschner, 1980; von Graevenitz, 1980; Yu, 1979).

## Ecology

For a long time, the confused status of *Serratia* taxonomy prevented any precise knowledge of the habitat of *Serratia* species. After the aforementioned *Serratia* species had been defined, however, it became apparent that they occupied different habitats. Table 1 shows the distribution of the major species of the *Serratia* strains isolated from small mammals and their territories, and from water, plants, and hospitalized human patients. Table 2 shows the distribution of the major biotypes of the *S. marcescens* strains isolated from these same habitats.

### *Serratia* in Water and Soil

Water is probably the principal habitat of *S. plymuthica*. Nomen species resembling *S. plymuthica* (Breunig’s Kiel bacillus, “*Bacillus miniaceus*,” “*Serratia miquelii*,” and “*S. esseyana*”) have also been isolated from water. Sea-water isolates belong to the same species as terrestrial water isolates. “*Serratia marinorubra*”

Table 1. Percent distribution among *Serratia* species of 1,543 strains isolated from different habitats.

Species	Percentage of isolates in habitat				
	Small mammals <sup>a</sup> (92 isolates)	Animal territories <sup>b</sup> (51 isolates)	Water (155 isolates)	Plants <sup>c</sup> (137 isolates)	Hospitalized patients (1,108 isolates)
<i>S. marcescens</i>	10	2	75	10	97
<i>S. plymuthica</i>	4	2	1	5	0
<i>S. liquefaciens</i>	43	55	11	38	2
<i>S. proteamaculans</i>	39	29	8	32	0
<i>S. grimesii</i>	3	10	5	7	0.5
<i>S. rubidaea</i>	0	0	0	1	0.2
<i>S. odorifera</i>	0	0	0	4	0.1
<i>S. ficaria</i>	0	2	0	4	0
Total percentage	100	100	100	100	100

<sup>a</sup>Rodents and shrews.

<sup>b</sup>From plants and soil around the traps where small mammals were captured.

<sup>c</sup>All plants except figs and coconuts.

Table 2. Percent distribution among *Serratia marcescens* biogroups of 1,210 strains isolated from four different habitats.

Biogroup	Percentage of isolates in habitat		
	Water (107 isolates)	Plants and rodents (37 isolates)	Hospitalized patients (1,066 isolates) <sup>a</sup>
A1	12	19	0.1
A2/6	23	5	7 <sup>b</sup>
A3	21	38	7
A4	34	38	26
A5/8	7	0	47
TCT	3	0	13
Total	100	100	100

<sup>a</sup>Isolated at the Pellegrin Hospital (Bordeaux, France) from 1968 through 1975.

<sup>b</sup>In addition, 72 strains of biotype A2b were isolated from infants (mostly from feces) in the neonatology ward.

was not a true marine species, as it showed no sodium requirement (P. Baumann, personal communication). Many strains of *S. fonticola* have been isolated from well waters and springs (Gavini et al., 1979). In a systematic search for *Serratia* in river water, 150 strains were isolated and were distributed in the following species: *S. marcescens* (75%), *S. liquefaciens* (11%), *S. proteamaculans* (8%), *S. grimesii* (5%), and *S. plymuthica* (1%) (F. Agbalika, F. Grimont, and P.A.D. Grimont, unpublished observations). The selective medium used (caprylate-thallos agar) did not allow isolation of *S. fonticola*.

Strains producing the non-diffusible red pigment prodigiosin seem to be toxic to protozoa (Groscof and Brent, 1964), and this may be an ecological advantage in water and soil. However, it seems that pigmented bacteria are more often isolated from unpolluted water (from springs or wells) than from polluted water (river water downstream from cities).

In soil, *S. marcescens* might play a role in the biological cycle of metals by mineralizing organic iron and dissolving gold and copper (Parès, 1964). A mineralization role has also been attributed to cold-tolerant *Serratia* associated with low-moor peat (Janota-Bassalik, 1963).

### *Serratia* on Plants

*Serratia proteamaculans* was once isolated from a leaf spot disease of the tropical plant *Protea cynaroides* (the King Protea) (Paine and Stansfield, 1919). However, the experimental lesions caused by *S. proteamaculans* on detached leaves of *Protea* suggest a hypersensitivity reaction (Paine and Berridge, 1921). Similar lesions were obtained with other species of *Serratia* (Grimont et al., 1978b). Inoculation of *S. marcescens* ICPB 2875 and *S. marinobrunnea* ICPB 2881 on tobacco and bean leaves also produced a typical hypersensitivity reaction (Lakso and Starr, 1970). A root disease complex of alfalfa (*Medicago sativa*) involving a *Fusarium* sp., a *Pseudomonas*

sp., and a bacterium named “*Erwinia amylovora* var. *alfalfae*” was observed by Shinde and Lukezic (1974). These isolates of “*Erwinia amylovora* var. *alfalfae*” were later identified as *Serratia marcescens* biotype A4a (Grimont et al., 1981).

Characteristic *Serratia* populations were found in figs. *S. ficaria* was recovered from most figs collected in California, Tunisia, France (Grimont et al., 1979c), Sicily (Giammanco and Amato, 1982; Grimont and Deval, 1982), and Greece (P.A.D. Grimont, unpublished observations). This species could be associated with *S. marcescens* (biogroups A1, A2, or A3) or other genera. Figs of the Calimyrna variety (Smyrna variety adapted to California) deserve special mention. Calimyrna figs contain only pistillate flowers and in order to become edible, they need to be pollinated specifically by the fig wasp *Blastophaga psenes*. The fig wasp has a life cycle limited to the caprifig, an inedible fruit produced by the caprifig tree. When a young female fig wasp makes her way out of a caprifig, she covers herself with pollen and a specific fungal and microbial flora (Phaff and Miller, 1961). The fig wasp will then enter an unpollinated caprifig of a new crop, pollinate it, oviposit in a pistillate flower, and die; or it may be carried by the wind to a Calimyrna fig—a cul-de-sac in the wasp life cycle—where the fig wasp will deposit pollen and the microbial/fungal flora in its desperate attempt to oviposit. Since 1927 (Caldis, 1927; Phaff and Miller, 1961; Smith and Hansen, 1931), a red-pigmented and a nonpigmented *Serratia* have been repeatedly isolated from caprifigs, pollinated Calimyrna figs, and fig wasps. The pigmented and nonpigmented isolates were identified as *Serratia marcescens* biotype A1b and *S. ficaria*, respectively (Grimont et al., 1979c; Grimont et al., 1981). The fact that the Calimyrna fig is internally sterile until the fig wasp enters it provides an ecological niche for these *Serratia* strains. The multiplication of *Serratia* in caprifigs or Calimyrna figs is limited, and these organisms do not

cause fig spoilage. Washing of a syconium cavity can yield 10 to 300 colony-forming-units (CFU) of *S. ficaria*.

*Serratia rubidaea* has been repeatedly isolated from coconuts bought in France (originating mostly from Ivory Coast) and in California (Grimont et al., 1981). The three subspecies (former biotypes B1 to B3) have been associated with coconuts, and when present, *S. rubidaea* numbered  $3 \times 10^4$  to  $6 \times 10^6$  CFU per gram of coconut milk or flesh (Bollet et al., 1989). It is believed that coconut palms have been disseminated worldwide from an Indo-Pacific origin. It would thus be interesting to compare the bacterial flora of coconuts collected in diverse countries.

In the course of an ecological survey and using the selective caprylate-thallos agar medium, *Serratia* species were frequently found associated with plants (Grimont et al., 1981). With respects to the presence of *Serratia*, plants (other than figs and coconuts) were classified into three prevalence groups. In the first group, which is composed of vegetables, mushrooms, mosses, and decaying plant material (i.e., wet plants), about 54% of the samples carried *Serratia* in counts varying from 2,000 to over 20,000 CFU per gram of plant (highest counts in mushrooms and leaves of radish, lettuce, cauliflower, and brussels sprout). In the second group, which is composed of grasses, *Serratia* prevalence was about 24%. In the third group composed of trees and shrubs, 8% of the samples (leaves) contained *Serratia* (10 to 300 CFU per gram).

*Serratia* strains isolated from plants other than figs and coconuts were distributed among eight *Serratia* species, although *S. liquefaciens* and *S. proteamaculans* were predominant (Table 1). The selective medium used (caprylate-thallos agar) was unable to support the growth of *S. fonticola*. *S. entomophila* was not isolated although this species can grow on the selective medium. Pigmented *S. marcescens* biotypes were rarely isolated from plants. Nonpigmented *S. marcescens* biogroups A3 and A4 were isolated from plants, but biogroups A5/8 and TCT were not (Table 2).

Vegetables used in salads might bring *Serratia* strains to hospitals and contaminate the patient's digestive tract. *S. marcescens*, *S. liquefaciens*, and *S. rubidaea* were found in 29%, 28%, and 11% (respectively) of vegetable salads served in a hospital in Pittsburgh (Wright et al., 1976). Similar results were found in a Paris hospital (Loiseau-Marolleau and Laforest, 1976). However, it still needs to be proven that biotypes/serotypes found in patients are the same as those found in salads. In the first described case of *S. ficaria* infection, the patient was very fond of figs (Gill et al., 1981).

## *Serratia* in Insects

There is an extensive literature on *Serratia* associated with insects. This topic has been reviewed in detail (Bucher, 1963a; Grimont and Grimont, 1978a; Steinhaus, 1959). The insects involved belong to numerous species and genera of the orders Orthoptera (crickets and grasshoppers), Isoptera (termites), Coleoptera (beetles and weevils), Lepidoptera (moths), Hymenoptera (bees and wasps), and Diptera (flies). Taxonomic uncertainties make a retrospective evaluation of the role of *Serratia* spp. in insect infections difficult. Red-pigmented *Serratia* were easily recognized (although not determined as to species with any certainty), whereas nonpigmented strains were often referred to genera other than *Serratia*. For example, *S. proteamaculans* or *S. liquefaciens* strains were named "*Bacillus noctuorum*" (White, 1923b), "*Bacillus melolonthae liquefaciens*" (Paillot, 1916), "*Paracolobactrum rhyncoli*" (Pesson et al., 1955), and "*Cloaca*" B type 71-12A (Bucher and Stephens, 1959); nonpigmented *S. marcescens* strains were named "*Bacillus sphingidis*" (White, 1923a) and "*Bacillus apisepticus*" (Burnside, 1928).

The distribution among the various *Serratia* species of 48 collection strains isolated from insects (Grimont et al., 1979b) showed a predominance of *S. marcescens* and *S. liquefaciens*. The absence of *S. plymuthica* (a pigmented species) in this collection is unexplained. Steinhaus (1941) reported the presence of *S. plymuthica* in the gut of healthy crickets (*Neombius fasciatus*), but the taxonomic schemes in vogue at that time did not allow a definite identification of *S. plymuthica*. The rarity of *S. rubidaea* (also a pigmented species) in insects might be explained by its inability to produce chitinase—a virulence factor for insect-associated *Serratia* species (Lysenko, 1976).

The red-pigmented *Serratia* isolates associated with the fig wasp *Blastophaga psenes* and formerly identified as *S. plymuthica* (Phaff and Miller, 1961) were reidentified as *S. marcescens* biotype A1b (Grimont et al., 1981). It is noteworthy that *S. ficaria* was isolated from both the fig wasp *Blastophaga psenes* and from a black ant, i.e., Hymenoptera (Grimont et al., 1979c).

*S. liquefaciens* and *S. marcescens* were found in sugar-beet root-maggot development stages (*Tetanops myopaeformis*), suggesting an insect-microbe symbiosis, as well as a nutritional interdependence (Iverson et al. 1984). A relationship appeared to exist between adult fly emergence and enzymatic chitin degradation of the puparium by the bacterial symbionts.

*S. marcescens* biotype A4b was repeatedly isolated from diseased honeybee (*Apis mellifera*) larvae in Sudan (El Sanoussi et al., 1987).



*Serratia marcescens*, *S. proteamaculans* and *S. liquefaciens* are considered potential insect pathogens (Bucher, 1960). They cause a lethal septicemia after penetration into the hemocoel. More than 70 species of insects were found to be susceptible to inoculation with *Serratia* (Bucher, 1963a). The lethal dose (LD<sub>50</sub>) of inoculated *Serratia* (by intrahemocoelic injection) was calculated for several insects 10–50 *Serratia* cells per grasshopper (Bucher, 1959), 5.1 cells per adult bollweevil (Slatten and Larson, 1967), 7.5 and 14.5 cells per third and fourth instar larva (respectively) of *Lymantria dispar* (Podgwaite and Cosenza, 1976), and 40 cells per *Galleria mellonella* larva (Stephens, 1959). The LD<sub>50</sub> of ingested *S. marcescens* is much higher. The hemolymph of insects—normally bactericidal for nonpathogens—cannot prevent multiplication of potential pathogens (Stephens, 1963). Lecithinase, proteinase, and chitinase play a role in the virulence of *Serratia* for insects, and purified *Serratia* proteinase or chitinase is very toxic when injected into the hemocoel (Kaska, 1976; Lysenko, 1976). *Serratia* strains in the insect digestive tract probably originate from plants. The multiplication of *Serratia* strains in the insect digestive tract has not been quantitatively studied. Antibacterial substances in ingested leaves might interfere with bacterial multiplication, but *Serratia* strains were found resistant to these (Kushner and Harvey, 1962). How potential pathogens such as *Serratia* can enter the hemolymph from the gut is generally unknown. However, spontaneous gut rupture, which happens in about 10% of grasshoppers, may allow *Serratia* strains to invade the hemocoel (Bucher, 1959). Direct injection occurs when *Itoplectis conquisitor* contaminated with *Serratia* stings host pupae to oviposit into their bodies (Bucher, 1963b). *Serratia* epizootics are common among reared insects (Bucher, 1963a). However, until recently, no genuine epizootic of *Serratia* infection among insects had been observed in the field.

Strains of *S. entomophila* and *S. proteamaculans* can be pathogenic for the grass grub *Costelytra zealandica*, which is a major pasture pest in New Zealand (Stucki and Jackson, 1984; Trought et al., 1982). Larvae of *Costelytra zealandica* feed on grass, clover, and other plant roots. Typically, their populations grow to a peak (about 600 larvae/m<sup>2</sup>) in 4 to 6 years after the pasture is sown and then collapse (to about 50 larvae/m<sup>2</sup>). Grass grub population collapse was found associated with the presence of a disease called amber disease (Trought et al., 1982). *S. entomophila* and *S. proteamaculans* were isolated from naturally infected larvae and shown experimentally to produce the disease when transmitted orally to healthy larvae (Grimont et al., 1988; Stucki and Jackson, 1984). The bacteria are ingested from

the soil. Infected larvae stop feeding within a few days, become translucent and then amber colored and lose weight until death occurs 4 to 6 weeks later. The bacteria colonize the gut and cause disease symptoms without invading the hemocoel. Field trials have shown that control of the grass grub was feasible by application of *S. entomophila* suspensions on pastures (Jackson and Pearson 1986). Reductions of 30 to 59% in the larval populations were obtained, with 47% of the remaining larvae being infected. Such bacterial treatment resulted in a 30% increase in grass production (dry matter).

### *Serratia* in Vertebrates

*Serratia* has been associated with chronic infections of cold-blooded vertebrates: nodular infection of *Anolis equestris*, the Cuban lizard (Duran-Reynals and Clausen, 1937); subcutaneous abscess of iguanid lizards (Boam et al., 1970); arthritis in the lizard *Tupinambis tequixin* (Ackerman et al., 1971); and ulcerative disease in the painted turtle *Chrysemys picta* (Jackson and Fulton, 1976). *Serratia* strains have also been recovered from the healthy, small, green pet turtle *Pseudemys scripta elegans* (McCoy and Seidler, 1973) and from geckos and turtles in Vietnam (Capponi et al., 1956).

Poultry may be contaminated with *Serratia*. A deadly *Serratia* epizootic among chick embryos was observed in a Japanese hatchery (Izawa et al., 1971). The hens carried *S. marcescens* in their digestive tract, but were themselves unaffected. Contamination of chicken carcasses with *S. liquefaciens* (Lahellec et al., 1975) and spoilage of eggs by red-pigmented *Serratia* (Alford et al., 1950) have been reported. A disseminated suppurative infection in a blue and gold macaw (*Ara ararauna*) affected with a chronic lymphoid atrophy has also been recorded (Quesenberry and Short, 1983).

*Serratia* strains (mostly red-pigmented) are responsible for 0.2–1.5% of cases of mastitis in cows (Barnum et al., 1958; Roussel et al., 1969; Wilson, 1963). Raw milk, therefore, may occasionally contain *Serratia* spp., and *S. liquefaciens* and *S. grimesii* are common in dairy products (Grimes and Hennerty, 1931). *Serratia* strains have been involved in septicemia in foals (Deom and Mortelmans, 1953), goats (Wijewanta and Fernando, 1970), and pigs (Brisou and Cadeillan, 1959); they have also been implicated in conjunctivitis of the horse (Carter, 1973) and abortion in cows (Smith and Reynolds, 1970). The isolation of *Serratia* from the anal sac of the red fox *Vulpes vulpes* (Gosden and Ware, 1976) has been reported.

*Serratia* strains have rarely been systematically searched for in the gut of animals. *S. fonticola*

was isolated in fecal samples from seven sparrows and unidentified birds. The study involved 90 wild European birds (Müller et al., 1986).

About 40% of trapped wild rodents and shrews carried *Serratia* strains in their gut without any visible sign of infection upon autopsy. The following animal species were found to carry *Serratia* spp.: 75/180, *Apodemus sylvaticus*; 7/23, *Microtus arvalis*; 3/11, *Clethrionomys glareolus*; 1/2, *Micromys minutus*; (rodents); 7/9, *Sorex*; and 1/3, *Crocidura* (shrews) (P. Giraud, F. Grimont, P.A.D. Grimont, unpublished observations). The *S. liquefaciens* complex (*S. liquefaciens*, *S. proteamaculans* and *S. grimesii*) represented 73 to 94% of all *Serratia* isolated from the gut of small mammals and from the soil and plants around traps (Table 1).

### *Serratia* in Humans

The healthy human being does not often become infected by *Serratia*, whereas the hospitalized patient is frequently colonized or infected. At present, *S. marcescens* is the only known nosocomial species of *Serratia* (Table 1). *S. liquefaciens* and *S. rubidaea* are occasionally isolated from clinical specimens, but their pathogenic role is not established. The isolation of other *Serratia* species is anecdotal (Farmer et al., 1985; Gill et al., 1981).

Clinically, *Serratia* infections do not differ from infections by other opportunistic pathogens (von Graevenitz, 1977): respiratory tract infection and colonization of intubated patients (e.g., Cabrera, 1969; von Graevenitz, 1980); urinary tract infection and colonization of patients with indwelling catheters (e.g., Maki et al., 1973); surgical wound infection or superinfection (e.g., Cabrera, 1969); and septicemia in patients with intravenous catheterization or complicating a local infection (osteomyelitis, ocular or skin infections) (e.g., Altmeier et al., 1969). Meningitis, brain abscesses, and intraabdominal infections are more exceptional.

The relationship between a *Serratia* strain and a patient may be in the form of an ephemeral association (in gut or throat, on hands or skin), a long-term colonization (in gut or urinary tract or on the skin), or a localized or generalized infection. The form of relationship might depend on the species or strain of *Serratia*, the entry route (ingestion, injection, catheter), an ecologic advantage (antibiotic treatment), or the patient's physiologic status. Patient factors have been reviewed by von Graevenitz (1977). The localization of a hospital-acquired infection is often determined by the kind of instrumentation or intervention done (i.e., the entry route). The same strain may cause a urinary infection in a urology ward, a bronchial colonization in an

intensive care unit, and a wound infection in a surgery unit. Five epidemiological situation models can be described (adapted from Farmer et al., 1976):

Model 1: "Endogenous," nonepidemic infections. Sporadic cases of infection are observed that are associated with different *Serratia* strains. The strains are often susceptible to several antibiotics. The presence of a *Serratia* strain in feces is not a sufficient proof of the endogenous origin of the infection (*Serratia* are probably ingested daily with food). There is no prevention mechanism in this epidemiological model.

Model 2: Common source epidemics. A single strain (species, biotype, serotype) is found to colonize or infect several patients. Any type of *Serratia* can be involved, including pigmented biotypes of *S. marcescens* or environmental species (e.g., *S. liquefaciens*, *S. grimesii*, *S. rubidaea*). The strain is often susceptible to several antibiotics. An investigation can reveal a common infection source such as a breathing machine (the nebulizer and tubings should be sampled), a batch of perfusion or irrigation fluid, or an antiseptic solution. Identification of the source usually allows efficient control of the epidemic.

Model 3: Patient-to-patient spread. The *Serratia* strain involved is typically a multiresistant member of a nonpigmented biogroup of *S. marcescens* (biogroups A3, A4, A5/8, or TCT). No common source is found although several secondary sources are possible (sink or sponge in patients' rooms, high rate of fecal carriage). Disinfection of inanimate sources often has no effect on the endemic state. In fact, the reservoir is usually the infected patient, and spread among patients occurs by transient carriage on the hands of nursing or medical staff. Handling of urinary catheters, wound drains, or tracheal tubes of infected (or colonized) patients contaminates the hands of personnel (Maki et al., 1973; Traub, 1972b). Hasty hand washing in an overbusy ward or in the course of an emergency (for example, in an intensive care unit) allows the transmission of the strain to uninfected patients. These patients are often immunocompromised, treated preventively with broad spectrum antibiotics and subjected to diverse instrumentation. The situation is typically endemic with epidemic peaks in periods of time when the ward is crowded. Transfer of infected or colonized patients from one ward to another or from one hospital to another often results in the spread of the outbreak to other wards or hospitals. Prevention of this epidemiological model is difficult (and in some countries, hopeless). Proper handwashing should be enforced. Some proposed solutions deal with ward/hospital management (e.g., smaller wards, higher nurse/patient ratio, separation of infected from

noninfected patients, and separation of their respective nurses).

**Model 4: Colonization of the newborn intestinal tract.** Typically, an investigation of a case of *Serratia* infection leads to the discovery that most newborns are colonized by a red-pigmented, drug-susceptible strain of *S. marcescens*. A common source can be identified ("sterile" water or oily antiseptic solution used to clean the baby's skin). Contamination may occur on the first day of life. Multiplication of the strain in soiled diapers may show a red discoloration (red-diaper syndrome). Control of this situation is sometimes difficult due to the size of the reservoir. Newly sterilized solutions are quickly contaminated again. Enforcement of handwashing and frequent sterilization (daily or more) of incriminated solutions may be helpful.

**Model 5: Pseudoepidemics.** A drug-sensitive strain of any *Serratia* species (e.g., *S. liquefaciens*) is unreproducibly isolated from several patients who show no sign of infection. Investigation of the plastic material used to "sterilely" collect blood occasionally allows the isolation of the environmental strain that contaminated the system (plastic tubing, EDTA, or citrate solution).

The above situations are sometimes mixed or less clear. References to reports fitting with the above models can be found in Farmer et al., 1976; Schaberg et al., 1976; von Graevenitz, 1977, 1980; and Daschner, 1980.

### Properties Relevant to Pathogenicity in Humans

*Serratia marcescens* is generally an opportunistic pathogen causing infections in immunocompromised patients. Among the possible pathogenicity factors found in *Serratia* strains are the formation of fimbriae, the production of potent siderophores, the presence of cell wall antigens, the ability to resist to the bactericidal action of serum, and the production of proteases.

In practice, each strain of the genus *Serratia* produces one to three different kinds of fimbrial hemagglutinin (HA) (Old et al., 1983). Five types of fimbriae have been observed in *serratiae*:

**Type 1 fimbriae:** thick, channelled fimbriae of external diameter 8 nm, associated with a mannose-sensitive hemagglutinin (MS-HA) reacting strongly with untanned fowl or guinea pig erythrocytes. The production of MS-HA is increased by serial, static broth cultures in air at either 20, 30, or 37°C. Production of MS-HA was found to be correlated with the ability of *S. marcescens* cells to attach to human buccal epithelial cells (Ismail and Som, 1982) or to the human urinary bladder surface (Yamamoto et al., 1985). This

type of HA was found to be produced by all (Old et al., 1983) or almost all (Franczek et al., 1986) *S. marcescens* strains, whether environmental or clinical, and in some strains of other *Serratia* species, except *S. plymuthica* and *S. fonticola*.

**Type 3 fimbriae:** thin, non-channelled fimbriae of external diameter 4–5 nm associated with a mannose-resistant hemagglutinin reacting with tannic acid-treated, but not fresh, oxen erythrocytes (MR/K-HA) (Old et al., 1983). MR/K-HA was found to be produced by almost all strains of all *Serratia* species studied by Old et al. 1983. However, Franczek et al. 1986 found MR/K-HA was more frequently produced by clinical than by environmental strains of *S. marcescens*. The MR/K-HA of all *Serratia* species, except *S. rubidaea*, were immunologically related. MR/K-HA from *S. rubidaea* was immunologically related to a *Klebsiella* MR/K-HA (Old et al., 1983).

**Thin fimbriae associated with a mannose-resistant hemagglutinin reacting with fowl, guinea pig, and horse erythrocytes (type FGH MR/P-HA).** This HA was produced by strains from all species except *S. plymuthica*, *S. odorifera*, and *S. fonticola* (Old et al., 1983). The corresponding fimbriae are immunologically related in the different species.

**Thin fimbriae associated with a mannose-resistant hemagglutinin reacting with fowl erythrocytes only (type F MR/P-HA).** This HA was produced by some *S. rubidaea* strains and was immunologically unrelated to other hemagglutinins (Old et al., 1983).

**Thick, channelled fimbriae of external diameter 9–10 nm:** associated with a mannose-resistant hemagglutinin reacting with fowl erythrocytes only (type F MR/P-HA). This HA was produced by some *S. fonticola* strains (Old et al., 1983).

Nearly all clinical or environmental *S. marcescens* strains produce potent siderophore(s) capable of scavenging iron from ethylenediamine di-O-hydroxyphenylacetic acid, a chelator with an association constant for ferric iron of  $10^{33.9}$  (Franczek et al., 1986). *Serratia* strains (*S. marcescens* and *S. liquefaciens* were tested) generally produce enterobactin (Reissbrodt and Rabsch, 1988) but only rarely produce aerobactin (Martinez et al., 1987). A novel iron (III) transport system named SFU, was evidenced in a *S. marcescens* strain. In this system, no siderophore production is involved (Zimmermann et al., 1989).

At least two patterns of hemolysis were found to be produced by *S. marcescens* on horse blood: a clear-cut narrow zone of hemolysis under the colony, evoking the action of a cell-bound hemolysin, and a fuzzier, diffusing zone of hemolysis evoking the production of a soluble hemolysin (Grimont and Grimont, 1978b). The

latter hemolysis pattern was produced only by *S. marcescens* biogroup A4.

The cell-bound hemolysin has been studied and direct contact between erythrocytes and *S. marcescens* cells has been demonstrated (Braun et al., 1985). The lysis of erythrocytes requires actively metabolizing bacteria and does not need calcium ions. A 7.3-kilobase-pair chromosomal fragment encoding the hemolytic activity has been cloned in *Escherichia coli* and sequenced (Poole et al., 1988). Two open reading frames designated *shlA* and *shlB* have been observed. The hemolysin protein (molecular weight 165,056) is encoded by *shlA*, and the product of *shlB* somehow activates *shlA*. Protein *shlA* integrates into the erythrocyte membrane and causes osmotic lysis through channel formation (Schiebel and Braun, 1989). When an *E. coli* strain carrying the *Serratia* hemolysin gene was compared to the isogenic strain without such a gene in an experimental rat model ( $10^7$  CFU/ml injected via urethra into the bladder), the strain carrying the hemolysin gene colonized the urinary tract more and led to a stronger inflammatory response compared to the hemolysin-negative strain (Marre et al., 1989).

A capsulated *Serratia* strain injected in the peritoneal cavity of the mouse multiplied and killed the mouse whereas an uncapsulated variant was avirulent (Ohshima et al., 1984).

Traub (1983) has shown that antibodies directed against O antigens of challenge strains afford passive protection in mice. The predominance of the O6 and O14 serotypes of *S. marcescens* in infections, the surface localization of LPS and its masking of other antigens suggests that LPS is a prime candidate for exploitation as a protective antigen in a vaccine against *S. marcescens* (Jessop, 1985).

Traub and Fukushima (1979a) classified *S. marcescens* strains into three categories with respect to their serum susceptibility. In solutions with 80% (vol/vol) of fresh serum, 88% of strains were found to be "delayed serum-sensitive" i.e., they were killed after a few hours exposure; 6% of strains were "promptly serum-sensitive" i.e., they were killed within a matter of minutes; and 6% of strains resisted an overnight exposure to serum. The delayed serum-sensitive strains were shown to be killed via the activation of the alternative pathway of the human complement system since killing was unaffected by inhibition of the classical pathway (Traub and Kleber, 1976). Conversely, promptly serum-sensitive strains were killed in a delayed fashion when the classical pathway was inhibited (Traub and Kleber, 1976). Depletion of fresh human serum of C3 with hydrazine hydrate completely abolished the bacterial activity against both promptly serum-sensitive and delayed serum-sensitive strains

(Traub and Fukushima, 1979a). Complement is of prime importance with respect to efficient opsonization and subsequent phagocytic killing by human peripheral blood granulocytes (Traub, 1982). In an intraperitoneal mouse model, treatment of animals with cyclophosphamide (generating a leukopenia) or zymosan (depletion of complement) did not increase susceptibility to *S. marcescens*. A combination of both treatments was necessary to significantly raise susceptibility to *S. marcescens* (Traub et al., 1983).

Crude culture filtrates have been shown to produce dermal hemorrhage after intradermal inoculation (Liu, 1961), corneal damage (Kreger and Griffin, 1975), and endophthalmitis after intravitreal injection (Salceda et al., 1973). These are due to one or more extracellular proteases produced by *S. marcescens*. Up to four proteases were purified (Lyerly and Kreger, 1979; Matsumoto et al., 1984)—two metalloproteases of 56 and 60 kDa and two thiol proteases of 73 kDa. These proteases play a prominent role in the pathogenesis of experimental pneumonia (Lyerly and Kreger, 1983) and keratitis (Lyerly et al., 1981). Most studies focused on the 56-kDa protease. Protease(s) were shown to cause: 1) liquefactive necrosis of the cornea (Kamata et al., 1985); 2) inactivation of five major proteinase inhibitors including  $\alpha_1$ -proteinase inhibitor (Virca et al., 1982),  $\alpha_2$ -macroglobulin, C1 inhibitor,  $\alpha_2$ -antiplasmin, and antithrombin III (Molla et al., 1989), which participate in regulating various cascade mechanisms (fibrinolysis and clotting cascade, complement system, inflammatory response); 3) degradation of immunoglobulins G and A, fibronectin, and other serum proteins (Molla et al., 1986; Traub and Bauer, 1985); 4) a potent toxic effect of fibroblasts that was mediated by internalization of a proteinase- $\alpha_2$ -macroglobulin complex via the  $\alpha_2$ -macroglobulin receptor, followed by regeneration of the proteinase activity in the cell (Maeda et al., 1987); 5) activation of the Hageman factor-kallikrein system generating kinin, which leads to enhanced vascular permeability (Matsumoto et al., 1984); and 6) cleavage of human C3 and C5 to yield leukotactic fragments (Ward et al., 1973).

Although resistance plasmids probably play no role in the virulence of *S. marcescens* in experimental models (Traub et al., 1983), multiple drug resistance may affect the course and prognosis of infections.

Non-pigmented strains of *S. marcescens* are generally more resistant to antibiotics than pigmented strains because they often harbor resistance plasmids. Environmental strains of *S. marcescens* or strains isolated before the antibiotic era are resistant to colistin, cephalothin, ampicillin (low level of resistance), tetracycline,

and nitrofurantoin and are usually susceptible to carbenicillin, third-generation cephalosporins, chloramphenicol, streptomycin, kanamycin, gentamicin, tobramycin, amikacin, trimethoprim/sulfamethoxazole, fosfomycin, nalidixic acid, and other quinolones (P.A.D. Grimont, unpublished observations). Clinical strains harboring plasmids often show additional resistance to various antibiotics (Farrar, 1980; Hedges, 1980), with a single plasmid being able to confer resistance to one to eleven antibiotics (Hedges, 1980; Olexy et al., 1982). Mutants resistant to nalidixic acid are often encountered in urology wards, and these mutants are often resistant to other quinolones. The antibiotics most often active against nosocomial strains of *S. marcescens* are amikacin, moxalactam, and cefotaxime. However, some strains may produce enzymes inactivating amikacin (Farrar, 1980) or may overproduce a class C  $\beta$ -lactamase (low-level resistance to cefotaxime and some other third-generation cephalosporins), which may combine with decreased permeability to  $\beta$ -lactams (high resistance to cefotaxime and some other third-generation cephalosporins) (Hechler et al., 1989).

## Compounds Produced by *Serratia*

### Pigments

Prodigiosin, a nondiffusible red pigment, is a secondary metabolite formed by the enzymatic condensation of 2-methyl-3-amylpyrrole (MAP) and 4-methoxy-2,2'-bipyrrole-5-carboxyaldehyde (MBC), leading to a tripyrrole derivative, 2-methyl-3-amyl-6-methoxyprodigiosene (Williams and Qadri, 1980). Little is known about the biosynthetic pathways of MAP or MBC, except that a proline molecule is incorporated intact into one of the pyrrole groups of MBC (Williams and Qadri, 1980). Pigment synthesis requires air, probably molecular oxygen. In the genus *Serratia*, prodigiosin is only produced by strains of *S. marcescens*, *S. plymuthica*, and *S. rubidaea*. In *S. marcescens*, prodigiosin is produced by biogroups A1 and A2/6 and never by biogroups A3, A4, A5/8, or TCT (Grimont, P. A. D., 1977). Nonpigmented strains of biogroups A1 or A2/6 are often blocked in the synthesis of either MAP or MBC (Grimont, P. A. D., 1977; Williams and Qadri, 1980). Strains in the nonpigmented biogroups are likely to lack the condensing enzyme (Ding and Williams, 1983). Some genes encoding prodigiosin biosynthesis were cloned by Dauenhauer et al. 1984 and expressed in *Escherichia coli*. The clones obtained had acquired the ability to condense MAP with MBC or to produce MAP in addition to the condensing enzyme.

Strains belonging to the ubiquitous biotype A4 can synthesize a pink diffusible pigment called

pyrimine (Grimont and Grimont, 1984; Williams and Qadri, 1980). Pyrimine, or ferrosamine A, is a ferrous complex of L-2-(2-pyridyl)-D'-pyrroline-5-carboxylic acid, a secondary metabolite also known to be produced by *Erwinia rhapsodica* (Feistner et al., 1983).

A yellow diffusible pigment, 2-hydroxy-5-carboxymethylmuconic acid semialdehyde, is produced from the *meta* cleavage of 3,4-dihydroxyphenylacetic acid (3,4-DHP) by the enzyme 3,4-DHP 2,3-dioxygenase (Trias et al., 1988). This enzyme is induced by tyrosine in all *S. marcescens* strains. However, presently only *S. marcescens* strains of biotype A8a, which have lost the ability to grow on aromatic compounds, can produce the yellow pigment. An uncolored muconic acid,  $\beta$ -*cis-cis*-carboxymuconic acid, can be produced by the *ortho* cleavage of 3,4-dihydroxybenzoate.

### Biosurfactants

Both pigmented and some nonpigmented strains of *Serratia marcescens* produce a biosurfactant that can act as a wetting agent. This wetting agent is produced in large amounts at 30°C but not at 37°C during the stationary phase of growth. Three aminolipids, designated W1 to W3 and having the wetting activity, were separated by thin-layer chromatography (Matsuyama et al., 1986). W1 was identified as serratamolide, a cyclodepsipeptide earlier discovered by Wasserman et al. 1961.

### Fatty Acids

The major fatty acid components in whole-cell methanolsates of *Serratia* species are 3-hydroxy-tetradecanoic, *n*-hexadecanoic, hexadecanoic, and octadecanoic (not separated from octadecadienoic) acids. These contribute 50–80% of the component fatty acids in each strain. Significant quantities are also contributed by *n*-tetradecanoic acid (Bergan et al., 1983).

### Flavors

Alkyl-methoxypyrazines are responsible for the potato-like odor produced by all strains of *S. odorifera* and *S. ficaria* and some strains of *S. rubidaea*. The major alkyl-methoxypyrazine produced by these *Serratia* strains is 3-isopropyl-2-methoxy-5-methylpyrazine. Minor compounds include 3-*sec*-butyl-2-methoxy-5(6)-methylpyrazine and 3-isobutyl-2-methoxy-6-methylpyrazine (Gallois and Grimont, 1985). Production of these three molecules by other bacterial species has never been observed. The food industry uses pyrazines to improve the flavor of some products.

## Enzymes

The most probable potential use of chitinase is in the treatment of the chitin-containing wastes produced by the seafood packing industry, bio-control agents against plant pathogenic fungi, production of adhesives and wound dressings, heavy metal recovery from waters, delayed-release agrochemicals or drugs, and dialysis membranes (Joshi et al., 1989). *S. marcescens* produces five proteins (molecular masses of 57, 52, 48, 36, and 21 kDa) with chitinolytic activity (Fuchs et al., 1986) whereas *S. liquefaciens* produces three proteins (molecular masses of 56, 51, and 36 kDa) with such activity (Joshi et al., 1989). The sequences of two *S. marcescens* chitinase genes, *chiA* encoding the 57 kD enzyme and *chiB* encoding the 52 kD enzyme, have been published (Jones et al., 1986; Harpster and Dunsmuir, 1989).

## Biotechnology

*Serratia* strains have been used in whole-cell bioconversion processes. Resting-cell suspensions of *S. marcescens* are able to convert vanillin to vanillic acid. At high substrate concentration (0.3%), 75% vanillin is converted (Perestello et al., 1989). 2,5-Diketogluconic acid, a key intermediate in the synthesis of ascorbic acid, can be produced from glucose at 20°C by *S. marcescens* (without requirement for a cofactor) and by *S. liquefaciens* and *S. grimesii* when supplied with the cofactor, pyrroloquinoline quinone (Bouvet et al., 1989).

Some genetic tools are available to construct *S. marcescens* strains of biotechnological interest. These tools include general transduction (Matsumoto et al., 1973), transformation (Reid et al., 1982), and use of nuclease-deficient, antibiotic-sensitive, and restrictionless mutants (reference is not an exact match Takagi and Kisumi, 1985). Recombinant strains obtained were histidase-less regulatory mutants producing L-histidine (Kisumi et al., 1977), L-arginine-producing mutants (Kisumi et al., 1978), an isoleucine-producing strain (Komatsubara et al., 1980), and a threonine-hyperproducing strain (Komatsubara et al., 1983).

## Isolation

### Underlying Principles

Pigmented species and biotypes of *Serratia* often exhibit pink or red colonies on nutrient agar. Use of low-phosphate agar without glucose, such as peptone-glycerol agar (Difco peptone,

5 g; glycerol, 10 ml; Difco agar, 20 g; distilled water, 1 liter), is best in order to demonstrate pigmentation (Williams and Hearn, 1967). Although the genus *Serratia* includes all the red-pigmented enterobacteria, identification of a pink or red colony should be confirmed by biochemical tests because a few nonenterobacteria may also produce prodigiosin (see "Introduction"). On nutrient agar, nonpigmented species or biotypes of *Serratia* give opaque-whitish, mucoid, or transparent smooth colonies. None of these traits is specific for the isolation of *Serratia* from a mixture of enteric bacteria. Colonies of *S. odorifera* and *S. ficaria* and occasionally *S. rubidaea* give off a specific, potato-like odor. Detection of this odor may suggest the presence of at least one colony of that species on the plate.

The salt tolerance and relatively low minimal growth temperature of all *Serratia* species may help devise a means of enriching them. A nutrient broth containing 4% NaCl, incubated at 15–20°C, will allow multiplication of *Serratia* but not of *Enterobacter cloacae* or *Aeromonas hydrophila* (P. A. D. Grimont, unpublished observations). This procedure has been used by us, but no systematic study of its effectiveness has been conducted.

Strains of the genus *Serratia* do not normally require addition of growth factors to a minimal medium. Thus, an enrichment medium for *Serratia* can be a minimal medium with a carbon source that is commonly used by *Serratia* spp. but not by non-*Serratia* spp.

Production of extracellular gelatinase, lecithinase, and DNase are essential characteristics of the genus *Serratia* (Ewing, 1986; Grimont et al., 1977b), *S. fonticola* excepted. Agar media that show the presence of one or a combination of extracellular enzymes can be used to differentiate *Serratia* colonies.

Finally, *Serratia* spp. are resistant to several compounds, including colistimethate, cephalothin (Greenup and Blazevic, 1971), and thallium salts (Starr et al., 1976), and one of these compounds can be used to make enrichment or differential media selective for *Serratia*. However, some *S. plymuthica* or *S. rubidaea* strains may be more susceptible to colistimethate, cephalothin, or ampicillin than is *S. marcescens*.

No medium has yet been devised to selectively isolate one particular *Serratia* species (other than *S. marcescens*), although specific carbon sources or conditions are listed in taxonomic papers (Grimont et al., 1977b). Occasionally, a selective medium may be devised according to a specific pattern of antibiotic resistance displayed by a nosocomial strain of *S. marcescens* (Denis and Blanchard, 1975), but such media cannot be of general utility.

### Selective Media Based on DNase Production and Antibiotic Resistance

Farmer et al. 1973 proposed the following deoxyribonuclease-toluidine blue-cephalothin (DTC) agar.

DTC Agar for Isolating *Serratia* (Farmer et al., 1973)

Deoxyribonuclease test agar (BBL)	21 g
Agar (Difco)	2.5 g
Toluidine blue O	0.05 g
Distilled water	500 ml

Mix on a mechanical stirrer until the dye dissolves, autoclave with a Teflon stirring bar at 121°C for 15 min, cool to 50°C, add 5 ml (500 mg) of cephalothin (Keflin injectible, Eli Lilly), stir and dispense in sterile petri dishes.

Typically, *Serratia* spp. grow on this blue DTC medium producing a red halo extending several millimeters around the colonies. “False negatives” (no growth or no halo) are very rare among *S. marcescens* isolates, but several strains of *S. rubidaea* and *S. plymuthica* failed to give the proper reaction (Starr et al., 1976).

A medium with DNase test agar, toluidine blue, cephalothin (30 µg/ml and colistimethate (30µ/ml) has been proposed by Cate (1972); and a medium with DNase test agar, toluidine blue, egg yolk, and cephalothin (100µ/ml) has been proposed by Goldin et al. 1969. The latter combines DNase and lecithinase detection.

Berkowitz and Lee (1973) devised the following medium for the isolation of *S. marcescens*:

DNase Medium for Isolating *Serratia marcescens* (Berkowitz and Lee, 1973)

Deoxyribonuclease test agar with methyl green (Difco)	42 g
L-Arabinose	10 g
Phenol red	0.05 g
Methyl green, 1%	4 ml
Distilled water	up to 1 liter

After autoclaving, add ampicillin (5 µg/ml), colistimethate (5 µg/ml), cephalothin (10 µg/ml), and amphotericin B (2.5 µg/ml).

*Serratia* colonies hydrolyze DNA, and the green component of the medium’s dark color disappears around the colonies. *S. marcescens* (or *S. entomophila*), unable to ferment L-arabinose, will give colonies surrounded by a red halo, whereas other *Serratia* species will give a yellow halo. The other *Serratia* species, however, may be inhibited by the antibiotic mixture. Wright et al. 1976 used this medium to isolate and enumerate *S. marcescens* from vegetable salads.

### Selective Media Based on Carbon-Source Utilization

A minimal medium with *meso*-erythritol as the sole carbon source was devised by Slotnick and Dougherty (1972). A modification of this

medium that included the antiseptic “Irgasan” (4’,2’,4’-trichloro-2-hydroxydiphenylether), has been proposed (Lynch and Kenealy, 1976). Unfortunately, *Serratia liquefaciens*, *S. plymuthica*, *S. entomophila*, *S. proteamaculans* biotypes Clc and RB, *S. odorifera* biotype 1, and the nosocomial biotypes A5, A8a, A8b, A8c and TCT of *S. marcescens* cannot grow with erythritol as sole carbon source (Grimont et al., 1977b; Grimont et al., 1978a; Starr et al., 1976). Therefore, these media should not be used in epidemiological or ecological surveys unless the study is knowingly limited to erythritol-positive serratiae.

A minimal medium with *meso*-inositol as sole carbon source has been proposed for the selective isolation of *Klebsiella pneumoniae* and *Serratia* spp. (Legakis et al., 1976). However, only a few genera and species have been studied by these authors, and it has been shown (Grimont et al., 1977b) that *Enterobacter aerogenes*, *E. cloacae*, *Erwinia herbicola*, and pectinolytic *Erwinia* spp. can also grow on a minimal medium with inositol as sole carbon source.

A caprylate-thallos (CT) agar medium has been devised for the selective isolation of all *Serratia* species and biotypes. This CT agar, derived from M70 minimal medium (Véron, 1975), is made up as follows (Starr et al., 1976):

CT Agar for Selective Isolation of *Serratia* (Starr et al., 1976)

First, a trace element solution (Véron, 1975) is prepared:

H <sub>3</sub> PO <sub>4</sub>	1.96 g
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.0556 g
ZnSO <sub>4</sub> ·4H <sub>2</sub> O	0.0287 g
MnSO <sub>4</sub> ·4H <sub>2</sub> O	0.0223 g
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025 g
Co(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	0.003 g
H <sub>3</sub> BO <sub>3</sub>	0.0062 g
Distilled water	1 liter

Store at 4°C (keeps well for at least 1 year). Then the following solutions (solution A and solution B) are prepared:

Solution A:

CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.0147 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.123 g
KH <sub>2</sub> PO <sub>4</sub>	0.680 g
K <sub>2</sub> HPO <sub>4</sub>	2.610 g
Trace element solution (see above)	10 ml
Caprylic acid	1.1 ml
Yeast extract (Difco) (5% wt/vol solution)	2 ml
Thallos sulfate	0.25 g
Distilled water	up to 500 ml

The pH is adjusted to 7.2 with NaOH. Autoclave at 110°C (or 120°) for 20 min.

Solution B:

NaCl	7 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1 g
Agar (Difco)	15 g
Distilled water	500 g

The pH is adjusted to 7.2. Autoclave at the same time as solution A. After autoclaving, solutions A and B are mixed aseptically, and the resulting medium is poured in thick layers into sterile, plastic petri dishes (25–30 ml for petri dishes 9–10 cm in diameter). The medium is no longer effective if it is remelted, but plates of CT agar keep well for several weeks at 4°C if contamination and desiccation are prevented.

CT agar is very useful for the isolation of *Serratia* spp. (except *S. fonticola*) from feces, sputum, and any other polymicrobial clinical sample. *Serratia* colonies are apparent within 3 days, and further incubation allows colonies grow (2–5 mm). Occasionally, *Providencia* spp., *Acinetobacter* spp., or *Pseudomonas* spp. may develop colonies on this medium. Other bacteria give only pinpoint colonies. When samples are from the natural environment or food, the results are less clear, especially if the sample contains nutrients. Several colonies must then be checked for DNase. Preenrichment in nutrient broth with 4% NaCl, incubated overnight at 20°C, can be used; about 0.1 ml of the enrichment culture is streaked onto CT agar. Broth that has supported overnight bacterial growth usually does not contain enough nutrients to adversely affect the selective isolation of *Serratia* spp. (P. A. D. Grimont, unpublished observations).

## Identification

The usual methods for the identification of serratiae and other Enterobacteriaceae can be found in Ewing (1986). However, following the pioneering example of Stanier et al. (1966) with the pseudomonads, developments in enterobacterial taxonomy (Bouvet et al., 1985; Grimont and Ageron, 1989; Grimont et al., 1977b; Grimont et al., 1988) have demonstrated the usefulness of carbon source utilization tests. Also, it should be noted that utilization tests are preferable to fermentation tests since strains able to utilize a polyalcohol sometimes fail to produce enough acid products to give a positive reaction in fermentation tests. Although all species can be identified with carbon source utilization tests, some conventional tests can also be used: tetrathionate-reduction and  $\beta$ -xylosidase tests are useful in clinical identification (Le Minor et al., 1970; Brisou et al., 1972). The methodology of carbon source utilization tests and other procedures that are not in general use, but are essential to *Serratia* identification, are detailed herein.

### Conditions of Incubation

Best results are obtained when *Serratia* cultures are incubated at 30°C. At 37°C, pigmentation often fails to appear, and the Voges-Proskauer

test is often negative. *S. plymuthica* may even fail to grow at 37°C. Otherwise, there is little difference between the results of tests held at 30°C and those held at 37°C as far as *S. marcescens* is concerned.

### Carbon Source Utilization Tests

Two methods can be used for carbon utilization tests: a minimal agar medium or API strips (API System, La Balme-les-Grottes, France).

The minimal agar medium employs the same M70 medium (Véron, 1975) used in the caprylate-thallos selective agar (see the recipe), but omits the yeast extract, thallos sulfate, and caprylate from solution A. Neutralized carbon sources (the purest brand available) are added to solution A to give a 0.1% final concentration (weight of the anion/vol) in the complete medium (0.2% in the case of carbohydrates). Sterilization of the carbon source in aqueous solution is achieved by filtration through membrane filters or by heating at 80°C for 20 min. Plates are examined for growth after 4 days (early reading) and 14 days (late reading).

The API strip method uses API 50 CH (containing 49 carbohydrates), API 50 AO (49 organic acids), and API 50 AA (49 aminoacids) galleries and a minimal medium containing growth factors provided by the manufacturer. These were described by Gavini et al. (1980). Since about one-third of the carbon sources are useless in the study of the Enterobacteriaceae, we use special galleries containing 99 selected carbon sources (including new compounds not included in the above-mentioned galleries). The inoculated galleries are examined for growth after 2 days (early reading) and 4 days (late reading). These galleries have been used for the description of several bacterial groups (Bouvet et al., 1985; Grimont et al., 1988; Grimont and Ageron, 1989).

In our experience, carbon source utilization tests (CSUT) gave reproducible and clear-cut results. Utilization of carbohydrates and polyalcohols often give more useful results than fermentation tests (FT). More than 95% of CSUT and FT using D-xylose, L-arabinose, trehalose, sorbitol, sucrose, and rhamnose gave the same results (P. A. D. Grimont, 1977). However, results with CSUT and FT are not parallel when adonitol, inositol, cellobiose, and lactose are studied. Whereas almost all *Serratia* strains can grow on inositol, acid from inositol was produced by only 76% of *S. marcescens*, 36% of *S. rubidaea*, 92% of *S. liquefaciens*-*S. proteamaculans* and 35% of *S. plymuthica* strains within 2 days. CSUT with adonitol is a very good test for separating *S. marcescens* from *S. liquefaciens* (99% vs. 0% positive strains, respectively), but



FT with adonitol is a poor test for separating these two species (32% vs. 0% positive strains in 2 days, respectively).

### Glucose Oxidation Test

The Lysenko test (Lysenko, 1961) was extensively modified by Bouvet et al. 1989. A 1-liter portion of glucose oxidation medium is composed of basal medium containing 8 g of nutrient broth (Difco), 0.02 g of bromocresol purple, and 0.62 g of  $MgSO_4 \cdot 7H_2O$  (2.5 mM). To 9.2 ml of autoclaved (121°C, 15 min) basal medium is added 0.4 ml of a filter-sterilized 1 M D-glucose solution (final concentration, 40 mM). This medium is supplemented with 0.4 ml of a fresh, sterile 25 mM iodoacetate solution (final concentration, 1 mM). The glucose oxidation medium is distributed (0.5 ml portions) into glass tubes (11 by 75 mm), which are plugged with sterile cotton wool. Bacteria grown overnight at 20 or 30°C on tryptocasein soy agar (Diagnostics Pasteur, Marnes-la-Coquette, France) supplemented with 0.2% (wt/vol) D-glucose are collected with a platinum loop, suspended in a sterile 2.5 mM  $MgSO_4$  solution and adjusted to an absorbance (at 600 nm) of about 4. Glucose oxidation medium is then inoculated with 50  $\mu$ l of bacteria suspension and vigorously shaken at 270 strokes per minute overnight at 20 or 30°C. The test is positive if a yellow color develop (acid production). Otherwise, the medium remains purple. When negative, the glucose oxidation test is repeated in the presence of 10  $\mu$ M pyrroloquinoline quinone (PQQ). The control strains used are the type strains of *S. marcescens* (positive without requirement for PQQ) and *S. liquefaciens* (positive only when PQQ is supplied) (Bouvet et al., 1989).

### Gluconate and 2-Ketogluconate Dehydrogenase Tests

The gluconate dehydrogenase test is performed as follows (Bouvet et al., 1989): Bacteria are grown overnight at 20°C on tryptocasein soy agar supplemented with 0.2% D-gluconate, then collected with a platinum loop and suspended in sterile distilled water to an absorbance (at 600 nm) of about 4. The reaction medium contains 0.2 M acetate buffer (pH5), 1% (wt/vol) Triton X-100, 2.5 mM  $MgSO_4$ , 75 mM gluconate, and (added immediately before use) 1 mM iodoacetate. A control medium contains the same ingredients except gluconate. The reaction medium and the control medium are dispensed (100  $\mu$ l portions) into 96-well microtiter plates (Dynatech AG, Denkendorf, Germany). Bacterial suspensions (10  $\mu$ l) are added to the reaction and the

control media, and the microtiter plates are incubated at 20°C for 20 min. Then, 10- $\mu$ l portions of a 0.1 M potassium ferricyanide solution (kept in the dark at room temperature for no longer than 1 week) are added to the wells, and the plates are gently shaken and incubated at 20°C for 40 min. Then 50  $\mu$ l of a reagent containing  $Fe_2(SO_4)_3$ , 0.6 g; sodium dodecyl sulfate, 0.36 g; 85% phosphoric acid, 11.4 ml; distilled water to 100 ml is added to each well. The plates are examined for the development of a green- to-blue color (due to Prussian blue) within 15 min at room temperature. The color in the uninoculated control medium remains yellow. The suggested control strains used are *Escherichia coli* K-12 (negative) and the type strain of *Serratia marcescens* (positive).

The 2-ketogluconate dehydrogenase test is the same as above, except that the control and reaction media are adjusted to pH 4.0, and 2-ketogluconate is used in place of gluconate in the reaction medium. Suggested control strains used are *Escherichia coli* K-12 (negative) and the type strain of *Serratia marcescens* (positive at 20°C, not at 30°C) (Bouvet et al., 1989).

### Tetrathionate Reductase Test

The tetrathionate reductase test was found to be very useful for the identification of *Serratia* spp. The liquid medium of Le Minor et al. (1970) is described below:

Tetrathionate Reductase Test for Identifying *Serratia* spp. (Le Minor et al., 1970)

$K_2S_4O_6$	5 g
Bromthymol blue (0.2% aqueous solution)	25 ml
Peptone water (peptone [Difco], 10 g; NaCl, 5 g; distilled water, 1 liter)	Up to 1 liter
Adjust pH to 7.4. Filter-sterilize and dispense 1 ml into 12-x-120-mm tubes. Within 1 or 2 days, reduction of tetrathionate will acidify the medium, indicated by a yellow color. Negative tubes remain green or turn blue.	

### Voges-Proskauer Test

Results of the Voges-Proskauer test are variable with some *Serratia* spp., probably because the end products of the butanediol pathway are metabolized (Grimont et al., 1977b). Many strains of *S. plymuthica* and *S. odorifera* give negative results with O'Meara's method (O'Meara, 1931) and positive results with Richard's procedure (Richard, 1972). In Richard's procedure, 0.5-ml aliquots of Clark and Lubs medium (BBL) are inoculated in 16-mm-wide test tubes. After overnight incubation at 30°C, 0.5 ml of  $\alpha$ -naphthol (6 g in 100 ml absolute ethyl alcohol) and 0.5 ml of 4 M NaOH are added. Positive tubes will turn red.

## Gas Production

Gas production differentiates *Serratia marcescens* from *S. liquefaciens*—*S. proteamaculans* better when glucose agar is used than when a liquid medium with a Durham inverted tube is used (Grimont et al., 1977b). Glucose agar consists of nutrient agar (meat extract [Liebig], 3 g; yeast extract [Difco], 10 g; agar [BBL], 15 g; distilled water to 1 liter; pH 7.4) supplemented with 1% (wt/vol) glucose. Dispense into tubes (160 × 10 mm). Autoclave 20 min at 120°C, cool at 50°C, and inoculate before the agar sets. Tubes are examined for bubbles of gas for up to 3 days.

## β-Xylosidase

The method of Brisou et al. (1972) for demonstrating β-xylosidase can conveniently be adapted for use with sterile microculture plates. Aqueous (1% wt/vol) p-nitrophenyl-β-xyloside is dispensed into the wells in 0.05-ml amounts, followed by 0.05 ml of a fresh bacterial suspension in 0.25 M phosphate buffer, pH 7. Plates are examined for a yellow color after 24 h.

## Identification of *Serratia* at the Genus Level

Members of the genus *Serratia* share the characteristics defining the family *Enterobacteriaceae*. Only occasionally can a nitrate-negative strain be isolated. The properties that best define the genus *Serratia* are listed in Table 3. Although lipase activity on tributyrin or corn oil is listed, *S. odorifera* strains are only weakly lipolytic. A weak urease activity, lack of motility, or presence of a capsule are occasionally observed. *Serratiae* are clearly differentiated from *Klebsiella* spp., *Enterobacter aerogenes*, and *E. cloacae* by production of gelatinase, lipase, DNase, and by growth on caprate or caprylate as sole carbon source. Some soft-rot *Erwinia* spp. produce extracellular proteinase and DNase, but these strains are pectinolytic and lack glucose and gluconate dehydrogenases (Grimont et al., 1977b).

## Identification of *Serratia* Species

The characteristics best allowing identification of each *Serratia* species are given in Tables 4 and 5. Carbon source utilization tests are invaluable for unambiguous identification. Indole production by *S. odorifera* is not reproducibly observed when peptone water is used. A defined medium containing tryptophane (e.g., “urée-indole” medium, Diagnostics Pasteur, Marnes-la-Coquette, France; or the indole test in API 20E strips) gives consistently positive results with this species. With the use of classical tests (Ewing, 1986), *S. plymuthica* may be confused either with

Table 3. Characteristics defining the genus *Serratia*.<sup>a</sup>

Positive characteristics of all species of the genus <i>Serratia</i> <sup>b</sup>
Motile rods
Growth at 20°C in 1 day
Growth at pH 9
Growth with 4% NaCl
Growth on minimal medium without addition of growth factor
Acid from maltose
Acid from mannitol
Acid from salicin
Acid from trehalose
ONPG <sup>c</sup> hydrolyzed
Glucose oxidized to gluconate <sup>d</sup>
Gluconate oxidized to 2-ketogluconate
Carbon source utilization tests: <i>N</i> -acetylglucosamine, <i>cis</i> -aconitate, 4-aminobutyrate, citrate, fructose, galactose, galacturonate, gluconate, glucose, glucuronate, glycerol, <i>m</i> -inositol, 2-ketogluconate, L-malate, maltose, mannitol, mannose, putrescine, ribose, trehalose
Additional positive characteristics of <i>Serratia</i> species other than <i>S. fonticola</i> <sup>b</sup>
Voges-Proskauer test (Richard)
Lipase (tributyrin, corn oil)
Growth on caprylate-thallose agar
DNase
Proteinase(s)
Negative characteristics of all species of the genus <i>Serratia</i> <sup>b</sup>
Urease (Ferguson)
H <sub>2</sub> S from thiosulfate
Phenylalanine/tryptophane deaminase
Polygalacturonidase
Amylase (4-day reading)
β-Glucuronidase
Carbon source utilization tests: 5-aminovaleate, <i>m</i> -coumarate, ethanolamine, glutarate, histamine, L-sorbose, tryptamine
Sodium ion requirement
Anaerobic growth with KClO <sub>3</sub>
Additional negative characteristics of <i>Serratia</i> species other than <i>S. fonticola</i>
Acid from dulcitol
Carbon source utilization tests: dulcitol, 3-phenylpropionate
<sup>a</sup> Taken from Grimont et al., 1977b, and unpublished observations.
<sup>b</sup> More than 90% of the isolates of each species give the same reaction.
<sup>c</sup> ONPG, <i>ortho</i> -nitrophenyl-galactoside.
In the presence of pyrroloquinoline quinone.

*S. proteamaculans*, *S. liquefaciens*, *S. rubidaea*, or *Enterobacter agglomerans*. In the past, *S. plymuthica* had been identified as “atypical *S. liquefaciens*” (negative for lysine and ornithine decarboxylase) or atypical *S. rubidaea* (sorbitol positive). The species *S. odorifera* can also be recognized in a number of “rhamnose-positive *S. liquefaciens*” strains. Carbon source utilization

Table 4. Differential characteristics of *Serratia* species.

Characteristic	<i>S. marcescens</i>	"liquefaciens group" <sup>a</sup>	<i>S. plymuthica</i>	<i>S. rubidaea</i>	<i>S. odorifera</i>	<i>S. ficaria</i>	<i>S. entomophila</i>	<i>S. fonticola</i>
Red pigment	d	–	v	v	–	–	–	–
Potato-like odor	–	–	–	v	+	+	–	–
Good growth at 5°C	–	+	+	–	+	+	+	+
Good growth at 40°C	+	–	–	v	ND	ND	+	ND
Indole	–	–	–	–	+	–	–	–
Tetrathionate reduction	d	+	–	–	–	–	–	+
Gas from glucose agar	–	+	v	–	–	–	–	v
β-Xylosidase	–	–	v	+	+	v	–	+
Oxidation of: Glucose to gluconate (without cofactor) <sup>b</sup>	+	–	v	+	+	+	+	–
2-Ketogluconate to 2,5-diketogluconate	+	d	–	–	–	–	–	–
Acid from:								
Adonitol	v	–	–	+	(+)	+	+	+
L-Arabinose	–	+	+	+	+	+	–	+
Lactose	d	v	(+)	+	(+)	v	–	+
D-Melibiose	–	+	+	+	+	+	–	+
D-Raffinose	d	+	+	+	d	+	–	+
L-Rhamnose	–	–	–	–	+	+	–	v
D-Sorbitol	+	+	d	–	+	+	–	+
Sucrose	+	+	+	+	d	+	+	–
D-Xylose	–	+	+	+	+	+	d	d
Lysine decarboxylase	+	+	–	d	+	–	–	+
Ornithine decarboxylase	+	+	–	–	d	–	–	+
Arginine decarboxylase	–	d	–	–	–	–	–	–
Tween 80 hydrolysis	+	+	+	+	–	+	+	+
Carbon source utilization:								
Adonitol	+	d	–	+	+	+	+	+
L-Arabinose	–	+	+	+	+	+	–	+
D-Arabitol	–	–	–	+	–	+	d	+
L-Arabitol	+	–	–	–	+	+	d	+
Betaine	–	–	v	+	–	–	–	–
Dulcitol	–	–	–	–	–	–	–	+
meso-Erythritol	d	–	–	+	d	+	–	+
Maltitol	–	+	+	+	–	+	–	v
Melezitose	–	+	+	d	–	+	–	–
D-Melibiose	–	+	+	+	+	+	–	+
Palatinose	–	+	+	+	–	+	–	+
Quinate	d	–	+	(+)	–	+	d	–
L-Rhamnose	–	d	–	–	+	+	–	+
D-Sorbitol	+	+	v	–	+	+	–	+
Sucrose	+	+	+	+	d	+	+	–
D-Tartrate	–	–	–	d	d	–	–	v
Tricarallylate	–	–	–	d	–	–	–	v
Trigonelline	d	–	–	+	+	v	–	–

<sup>a</sup>The liquefaciens group or complex includes *S. liquefaciens*, *S. proteamaculans*, and *S. grimesii*.

Without addition of pyrroloquinoline quinone. All species produce gluconate from glucose in the presence of pyrroloquinoline quinone.

<sup>b</sup>Symbols: +, positive for 90% or more strains in 2-day reading; –, negative for 90% or more strains in 4-day reading; d, test used to differentiate biotypes; v, variable reactions; ( ), 4-day reading; ND, not determined.

tests can help to sort out these “atypical” strains: after examining more than 5,000 *Serratia* strains, we have found no *S. liquefaciens*, no *S. grimesii*, and no *S. plymuthica* strains that were able to grow on adonitol and erythritol and no *S. rubidaea* strains able to grow on sorbitol as sole carbon source.

### Identification of *Serratia* Biogroups and Biotypes

Biotypes in *Serratia marcescens*, *S. proteamaculans*, *S. plymuthica*, and *S. rubidaea* were first defined by numerical taxonomy (Grimont et al., 1977b). We recognize 18 biotypes in *S. marce-*

Table 5. Identification of species in the *Serratia liquefaciens* complex.<sup>a</sup>

Characteristic	<i>S. proteamaculans</i>						
	<i>S. liquefaciens</i> Clab	subsp. <i>proteamaculans</i>			subsp. <i>quinovora</i> RQ	<i>S. grimesii</i>	
		C1c <sup>b</sup>	EB	RB		C1d <sup>c</sup>	ADC
Growth on:							
trans-Aconitate	-	+	+	-	+	-	+
Adonitol	-	-	(+)	-	-	-	-
Benzoate	-	-	(+)	v	-	+	-
m-Erythritol	-	-	+	-	-	-	-
Gentisate	-	-	-	+	-	-	-
D-Malate	+	-	-	-	v	(v)	(v)
L-Rhamnose	-	-	-	+	d	-	-
m-Tartrate	+	-	-	-	d	-	-
Arginine decarboxylase	-	-	-	-	-	+	+
Esculine hydrolyzed	+	+	+	+	-	+	+

<sup>a</sup>Symbols as in Table 4.

<sup>b</sup>The type strain of *S. proteamaculans* (ATCC 19323) corresponds to biotype C1c.

<sup>c</sup>The type strain of *S. grimesii* (ATCC 14460) corresponds to biotype C1d.

The potato-like odor produced by *S. odorifera*, *S. ficaria*, and a few strains of *S. rubidaea*, was identified as a pyrazine (3-isopropyl-2-methoxy-5-methylpyrazine) (Gallois and Grimont, 1985).

Table 6. Identification of *Serratia marcescens* biotypes.<sup>a</sup>

Characteristic	Biotype																	
	A1		A2		A6	A3			A4		A8			TCT	TC	TT		
	a	b	a	b	a	a	b	c	d	a	b	A5	a				b	c
Growth on:																		
m-Erythritol	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	
Trigonelline	-	-	-	+	-	-	+	-	+	-	-	+	+	+	+	+	-	+
Quinate and/or 4-hydroxybenzoate	-	-	-	-	+	-	-	-	-	+	-	+	+	+	-	-	-	-
3-Hydroxybenzoate	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-
Benzoate	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Malate/m-tartrate <sup>b</sup>	+	-	+	+	v	-	-	v	-	+	+	+	v	-	-	+	+	-
Gentisate	-	-	+	+	+	+	+	v	-	+	+	-	v	+	v	-	-	-
Lactose	-	-	-	-	-	- <sup>c</sup>	-	- <sup>c</sup>	- <sup>c</sup>	- <sup>c</sup>	-	- <sup>c</sup>	-	- <sup>c</sup>	+	- <sup>c</sup>	- <sup>c</sup>	-
Tetrathionate reduction	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+
Red pigment	+ <sup>d</sup>	+ <sup>d</sup>	+	+	+ <sup>d</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-

<sup>a</sup>Symbols as in Table 4.

<sup>b</sup>Growth on D-malate and meso-tartrate is correlated with growth on DL-carnitine (Grimont, unpublished observations).

<sup>c</sup>Lactose-positive strains are occasionally isolated.

<sup>d</sup>Nonpigmented strains are occasionally isolated.

scens, 4 in *S. proteamaculans*, 2 in *S. grimesii*, 3 in *S. rubidaea*, 2 in *S. odorifera*, and 2 in *S. entomophila*. Characteristics of these biotypes are given in Tables 6–9. Carbon source utilization tests are major elements in the identification of these biotypes. Multipoint inoculation devices (e.g., Denley Multipoint Inoculator, Denley Instruments, Ltd., Bolney Cross, Bolney, Sussex, England), are the most useful for biotyping 20 or more strains at a time. Biotype identification is faster (1–4 days) when API strips containing carbon sources are used. Alternatively, filter paper disks impregnated with each carbon source can be deposited on an inoculated minimal agar

(Sifuentes-Osornio and Gröschel, 1987). Differentiation between *S. proteamaculans* biotypes Cla and Clb is superfluous; electrophoresis of proteinases (Grimont et al., 1977a) could not discriminate between these two biotypes.

### Serotyping of *Serratia marcescens*

The systematic inventory of somatic (O) antigens of *S. marcescens* began in 1957 (Davis and Woodward, 1957). Flagellar antigens (H) were described (Ewing et al., 1959) and an antigenic scheme was developed (Ewing, 1986; Ewing et al., 1959; Le Minor and Pigache, 1977, 1978; Le

Table 7. Identification of *Serratia rubidaea* biotypes.<sup>a</sup>

Trait	Biotype <sup>b</sup>		
	B1	B2	B3
Growth on:			
Histamine	v	–	+
D-Melezitose	–	+	+
D-Tartrate	+	–	v
Tricarallylate	–	v	–
Voges-Proskauer (O'Meara)	+	–	v
Lysine decarboxylase	+	+	–
Malonate (Leifson)	+	+	–

<sup>a</sup>Symbols as in Table 4.

<sup>b</sup>Biotype B1 corresponds to the subspecies designated as *S. rubidaea* subsp. *burdigalensis*; B2 to *S. rubidaea* subsp. *rubidaea*; and B3 to *S. rubidaea* subsp. *colindalensis*.

Unpublished observations, F. Grimont and P. A. D. Grimont.

Table 8. Identification of *Serratia odorifera* biotypes.<sup>a</sup>

Trait	Biotype	
	1 <sup>b</sup>	2
Growth on:		
<i>m</i> -Erythritol	–	+
L-Fucose	v	+
D-Raffinose	+	–
Sucrose	+	–
D-Tartrate	+	–
Ornithine decarboxylase	+	–
Acid from sucrose	+	–
Acid from raffinose	+	– <sup>c</sup>

<sup>a</sup>Symbols as in Table 4.

<sup>b</sup>The type strain corresponds to biotype 1.

<sup>c</sup>Some strains were positive in 3–7 days.

Table 9. Identification of *Serratia entomophila* biotypes.<sup>a</sup>

Trait	Biotype	
	1 <sup>b</sup>	2
Growth on:		
D-Arabitol	+	–
L-Arabitol	–	+
D-Malate	–	v
Quinate	+	v
D-Xylose	–	+

<sup>a</sup>Symbols as in Table 4.

<sup>b</sup>The type strain corresponds to biotype 1.

Biotyping of *S. marcescens* is epidemiologically useful (Grimont and Grimont, 1978b, Sifuentes-Osornio et al., 1986). Pigmentation occurs only in five *S. marcescens* biotypes: A1a, A1b, A2a, A2b, and A6. The 13 other *S. marcescens* biotypes correspond to nonpigmented strains: A3a, A3b, A3c, A3d, A4a, A4b, A5, A8a, A8b, A8c, TCT, TT, and TC. Biotypes TT and TC are rarely found and their ecological-epidemiological significance is unknown. Nonpigmented biotypes A3abcd and A4ab are ubiquitous, whereas nonpigmented biotypes A5, A8abc, and TCT seem restricted to hospitalized patients (these biotypes can however be isolated from sewage-polluted river water). Pigmented biotypes are ubiquitous.

Minor and Sauvageot-Pigache, 1981; Le Minor et al., 1983; Sedlak et al., 1965; Traub, 1981, 1985; Traub and Fukushima, 1979b; Traub and Kleber, 1977). The present system consists of 24 somatic antigens (O1 to O24) and 26 flagellar antigens (H1 to H26). Serotyping of *S. marcescens* is not easy. Cross-reactions occur between O antigens 2 and 3 (the common antigen is referred to as Co/2, 3); 6 and 7; 6, 12, and 14; and 12, 13, and 14 (the common antigen is referred to as Co/12, 13, 14). Some strains seem to have more than one O-factor (e.g., O3,21). Cross-reactions between O6 and O14 are so extensive that the epidemiological distinction between these two antigens (now referred to as O6/O14) was abandoned.

The accuracy of the O-agglutination test with boiled antigens has been questioned (Gaston et al., 1988; Gaston and Pitt, 1989a). Immunoblotting with electrophoresced lipopolysaccharide (LPS) antigens showed that O-agglutinating sera often reacted with a heat-stable surface antigen rather than with LPS. This heat-stable surface antigen masks the expression of O-specific LPS antigens. Gaston and Pitt (1989b) proposed a simple dot enzyme immunoassay (dot EIA) that could offer greater accuracy than agglutination tests for serotype identification. Most strains possess distinct acidic polysaccharides of microcapsular origin. Oxley and Wilkinson (1988a) have shown that the O13 antigen is a microcapsular, acid polymer, rather than an integral part of the lipopolysaccharide. No high-molecular weight LPS corresponding to O-side chain material was detected in O-serogroup reference strain O11 or O13 (Gaston and Pitt, 1989a). The O6/O14 antigen is a partially acetylated acidic glucomannan (Brigden and Wilkinson, 1985). Three different neutral polymers (O-side chain polysaccharides) have been found in three O14 strains. Each of these polymers has been shown to occur also in strains of serogroups O6, O8, or O12. The polymer with a disaccharide repeating-unit of D-ribose and 2-acetamido-2-deoxy-D-galactose present in the O12 reference strain, the O14:H9 reference strain, and in a O13:H7 reference strain all correspond to the Co/12,13,14 antigen shared by these strains (Brigden et al., 1985; Brigden and Wilkinson, 1983; Oxley and Wilkinson, 1988b).

To overcome the tediousness of measuring flagellar agglutination, an immobilization test in semisolid agar has been described for determination of H antigens (Le Minor and Pigache, 1977). The technique is facilitated by the use of pools of nonabsorbed sera. No anti-H:9 serum is needed in pools, as bacteria with H:9 antigen are immobilized by anti-H:8 and anti-H:10 sera. Once the unknown strain is immobilized by a serum pool, corresponding individual sera are

Table 10. Correlation between biogroups and serotypes in *Serratia marcescens*.

Biogroup <sup>a</sup>	Serotypes included
A1	O5:H2; O5:H3; O5:H13; O5:H23; O10:H6; and O10:H13
A2/6	O5:H23; O6,14:H2; O6,14:H3; O6,14:H8; O6,14:H9; O6,14:H10; O6,14:H13; O8:H3; and O13:H5
A3	O3:H5; O3:H11; O4:H5; O4:H18; O5:H6; O5:H15; O6,14:H5; O6,14:H6; O6,14:H16; O6,14:H20; O9:H9; O9:H11; O9:H15; O9:H17; O12:H5; O12:H9; O12:H10; O12:H11; O12:H15; O12:H16; O12:H17; O12:H18; O12:H20; O12:H26; O13:H11; O13:H17; O15:H3; O15:H5; O15:H8; O15:H9; O17:H4; O18:H21; O18:H26; O22:H11; and O23:H19
A4	O1:H1; O1:H4; O2:H1; O2:H8; O3:H1; O4:H1; O4:H4; O5:H1; O5:H6; O5:H8; O5:H24; O9:H1; O13:H1; O13:H11; and O13:H13
A5/8	O2:H4; O3:H12; O3,21:H12; O4:H12; O5:H4; O6,14:H4; O6,14:H12; O8:H4; O8:H12; O15:H12; and O21:H12
TCT	O1:H7; O2:H7; O4:H7; O5:H7; O5:H19; O7:H7; O7:H23; O10:H8; O10:H9; O11:H4; O13:H7; O13:H12; O16:H19; O18:H9; O18:H16; O18:H19; O19:H14; O19:H19; O20:H12; and O24:H6

<sup>a</sup>Biogroup A1 is composed of biotypes A1a and A1b; A2/6 is composed of A2a, A2b, and A6; A3 of A3a, A3b, A3c, and A3d; A4 of A4a and A4b; A5/8 of A5, A8a, A8b, and A8c; and TCT of TCT and TT.

used to achieve final identification of H antigens. The following absorbed sera need to be prepared: anti-H:8 absorbed by H:10; anti-H:10 absorbed by H:8; and anti-H:3 absorbed by H:10. Other cross-reactions are overcome by dilution of sera. The immobilization test works because H antigens are monophasic in *S. marcescens*.

A total of 175 complete (O+H) serotypes have been identified at this time. No plasmid or phage has yet been found to alter the serotype of a strain.

Some striking correlations between antigenic composition and biotype have been shown (Grimont et al., 1979a). Pigmented biotypes and non-pigmented biotypes are antigenically segregated. Serotyping subdivides the biotypes, but when two biotypes correspond to the same serotype, these biotypes usually differ by only one biochemical reaction. Biotypes can be united into "biogroups" in order to clarify biotype-serotype correlation (Table 10). These biogroups may be valid infraspecific taxa.

### Serotyping of Other Species

An antigenic scheme for *S. ficaria* is presently available (Grimont and Deval, 1982). Four somatic (O) and one flagellar (H) antigens were identified that defined four serovars. All American strains studied (isolated from the fig wasp or from a human patient) belonged to serotype O1:H1; strains from the Mediterranean region (Sicily, Tunisia, France) were not so antigenically uniform and all four serotypes were found in figs from Sicily.

An antigenic scheme for *S. rubidaea* has also been devised (Grimont et al., manuscript in preparation). Nine somatic and five flagellar antigens were identified. A total of 53 strains were distributed among 14 complete (O+H) serotypes.

### Bacteriocin Typing of *Serratia marcescens*

Two different schemes have been developed for typing *Serratia marcescens* strains by susceptibility to bacteriocins ("marcescins"). The system of Traub (1980) was developed since 1971 (Traub et al., 1971) and uses marcescins (incomplete phage structures) produced by 10 selected strains. In 1980, 73 bacteriocin types had been described and about 93% of strains were typable (Traub, 1980). In this system, bacteriocin type 18 is often associated with serotype O6/14:H12 in multiple-drug-resistant nosocomial strains.

Farmer (1972b) independently developed another system of typing based on susceptibility to marcescins. After mitomycin induction, 12 bacteriocin-producing strains were selected, and the 12 marcescins could subdivide 93 strains into 79 types.

Typing of *S. marcescens* by production of bacteriocin was also developed by Farmer (1972a). Twenty-three strains susceptible to bacteriocins were selected to serve as indicator strains. Each unknown *S. marcescens* strain is treated with mitomycin, and the marcescin produced (if any) is identified according to the pattern of susceptibility demonstrated by indicator strains. This method could be used to type 91% of the strains studied.

### Phage Typing of *Serratia*

Farmer (1975) has developed a phage typing system common to *Serratia marcescens*, *S. liquefaciens*, and *S. rubidaea* (*marinorubra*). With 74 phages, 95% of the *S. marcescens* strains and 50% of the *S. liquefaciens* and *S. rubidaea* strains could be typed. Phage typing was found to be convenient for subdividing prevalent serotypes (Negut et al., 1975). F. Grimont (1977) independently developed another phage typing system

for nosocomial strains isolated in Bordeaux. Most of these *S. marcescens* strains gave phage susceptibility patterns that corresponded to phage types described by F. Grimont (1977); however, strains isolated in other cities of France were less often typable, and strains received from other countries were rarely typable in this system.

### Typing by Enzyme Electrophoresis

By using agar gel electrophoresis, Grimont et al. (1977a) and Grimont and Grimont (1978c) detected seven different proteinases (called P<sub>5</sub>, P<sub>6</sub>, P<sub>7</sub>, P<sub>9a</sub>, P<sub>9b</sub>, P<sub>11</sub>, and P<sub>12</sub>) produced by strains of *S. marcescens*. Each *S. marcescens* strain can produce one to four proteinases; 651 strains of *S. marcescens*, isolated over a period of six years in a French hospital, gave 33 different proteinase patterns (called zymotypes). However, six zymotypes alone accounted for 76% of the isolates. Proteinase electrophoresis was thus used as an epidemiological marker (Grimont and Grimont, 1978c).

Goulet (1978, 1981) showed that the *Serratia* species were characterized by distinct electrophoretic patterns of their esterases. However, these esterase profiles were not used as epidemiological marker.

Enzyme polymorphism among 99 *S. marcescens* isolates was determined by electrophoresis of nine enzymes (Gargallo-Viola, 1989). More than one electromorph was found for seven of these enzymes (alanine dehydrogenase, catalase, NADP-dependent malic enzyme, 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, NADP-dependent glu-tamate dehydrogenase, and indophenol oxidase), and 33 distinctive electrophoretic types. One group was represented exclusively by isolates belonging to nonpigmented biotypes recovered almost entirely (97%) from clinical samples. The other group comprised all isolates belonging to a pigmented biotype. Electrophoresis of glucose-6-phosphate dehydrogenase alone can distinguish *S. marcescens* strains belonging to a pigmented biotype from those belonging to a nonpigmented biotype (Gargallo et al., 1987). A very good correlation was found between electrophoretic periplasmic protein patterns, enzyme electrophoresis, and biotyping by carbon source utilization (Gargallo-Viola and Lopez, 1990).

### Restriction Patterns

Electrophoretic patterns of fragments produced after cleavage of total DNA by restriction endonucleases have been used to compare *S. marcescens* strains in epidemiological studies (McGeer et al., 1990).

A new, generally applicable typing method has recently been proposed by Grimont and Grimont (1986) in which the DNA restriction fragments carrying rRNA genes are visualized after hybridization with a labelled *E. coli* 16+23S rRNA probe (rRNA gene restriction patterns). Five to seven DNA restriction fragments (after digestion by *Bam* HI) or 11 to 13 fragments (after digestion by *Eco*RI) were observed with all *Serratia* strains tested (Grimont and Grimont, manuscript in preparation). The different *S. marcescens* biogroups display different rRNA gene restriction pattern. This method should be useful in resolving occasionally uncertain (or conflicting) results given by serotyping and biotyping.

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## The Genera *Proteus*, *Providencia*, and *Morganella*

JIM MANOS AND ROBERT BELAS

### Introduction

The three genera *Proteus*, *Morganella* and *Providencia* presently comprise a total of ten species. All are motile, Gram-negative rods with peritrichous flagella, and are assigned to the *Enterobacteriaceae* family mainly on the basis of shared biochemical characteristics. Most significantly, they are characterized by their ability to oxidatively deaminate phenylalanine and, in most cases (except for some *Providencia* spp.), to hydrolyze urea (Farmer et al., 1977; Moltke, 1927; Wenner and Retger, 1919). Unusual features include the ability of *Proteus* sp. to differentiate into swarmer cells upon colonization of solid surfaces. This topic will be covered in more detail in ensuing sections.

Interest in the species comprising these genera has occurred mainly from a clinical perspective, as they include a number of significant human pathogens. In human disease, most infections are associated with prolonged hospitalization and in the case of *Proteus* and *Morganella* spp., colonization of indwelling catheters and associated urinary tract infections (UTIs).

### Taxonomy and Phylogeny

Before the advent of phylogenetically based classification, an array of biochemical tests formed the basis for taxonomic classification of the genera *Proteus*, *Providencia*, and after its separation into a new genus, *Morganella*. Tables 1 and 2 list the major biochemical tests used to compare and differentiate between the genera. As shown in Table 1, the most significant shared characteristics are the (oxidative) deamination of phenylalanine and tryptophan; both are used to distinguish between these three genera and other *Enterobacteriaceae* that do not produce these deaminases. The tests for production of these deaminases were developed in the 1950s and still are widely used (Henriksen, 1950; Thibault and Le Minor, 1957). Table 2 shows the biochemical tests that are commonly used to distinguish between these genera. The only test that will dis-

tinguish *Morganella* from *Proteus* and *Providencia* is the lysine iron agar test. On the other hand, several tests will distinguish *Proteus* from *Providencia*. *Providencia* is characterized by the production of acid from a variety of sugars, whereas *Proteus* is distinguished from *Providencia* by the hydrolysis of gelatin and the production of lipase and hydrogen sulfide. The use of molecular phylogenetic methods of classification has resulted in several species being reassigned to separate genera based on relatedness at the DNA level. These changes include: the new genus *Morganella*, with transfer of the species *Proteus morganii* to it (Brenner et al., 1978); the classification of *Providencia alcalifaciens* biogroup 3 as the separate species (*Providencia rustigianii*; Higashitani et al., 1995); and the identification of a subgroup within the latter as a distinct species, *Providencia heimbachae* (Muller et al., 1986b).

Isolated in 1906 (Morgan, 1906), *Morganella morganii* was originally included in the genus *Proteus* as *Proteus morganii*. Brenner et al. (1978) showed that *P. morganii* had less than 20% homology to *Proteus* and *Providencia* spp., necessitating relocation into a new genus. The method employed by Brenner et al. in this reclassification compared the amount of single stranded <sup>32</sup>P-labeled DNA reassociating with DNA from the same source (homologous reaction), relative to the amount reassociating with DNA from other species in these genera. The resulting value was expressed as “percent DNA relatedness.” *Morganella morganii* has subsequently been subdivided into two subspecies based on its ability to ferment trehalose and the results of DNA-DNA hybridization studies (Jensen et al., 1992). *Morganella morganii* subsp. *morganii* is composed of four biogroups (A, B, C and D), and it does not ferment trehalose, though *M. morganii* subsp. *sibonii* contains three biogroups (E, F and G) and does ferment trehalose.

Further subdivision of these species is likely with the application of more discriminatory molecular methods of characterization. As an example, the use of molecular typing by 16S ribosomal RNA (rRNA) gene fingerprints (ribotyping) has recently demonstrated that, though

Table 1. Biochemical characteristics common to the genera *Proteus*, *Morganella* and *Providencia*.

Biochemical test	<i>Proteus</i>	<i>Morganella</i>	<i>Providencia</i>
Arginine dihydrolase	–	–	–
Lysine decarboxylase	–	–	–
Ornithine deaminase	+	+	+
Phenylalanine deaminase	+	+	+
Growth on KCN	+	+	+
D-Glucose from acid	+	+	+
Acid from melibiose	–	–	–
Nitrite from nitrate	+	+	+
Oxidase production	–	–	–
ONPG production	–	–	–
Pectate utilization	–	–	–
Tartrate utilization	+	+	+

Symbols and Abbreviations: +, present; –, absent; KCN, potassium cyanide; and ONPG, *o*-nitrophenyl- $\beta$ -D-galactopyranoside.

clinical isolates of *P. mirabilis*, *P. penneri*, *M. morgani* and *P. heimbachae* had identical ribotyping patterns to those of their respective type strains, those from the remaining species all exhibited heterogeneity, containing from two to four ribogroups each (Pignato et al., 1999). Thus it is possible that subdivision of members of these latter species into one or more subspecies may be considered appropriate in the future.

The genus *Proteus* presently contains four species: *P. mirabilis*, *P. vulgaris*, *P. penneri* and *P. myxofaciens*. *Morganella* has one species (*M. morgani*), whereas *Providencia* contains five species (*Providencia stuartii*, *Providencia rettgeri*, *P. rustigianii*, *P. heimbachae* and *P. alcalifaciens*; (Rozalski, 1997; Rustigian, R., and C. A. Stuart, 1945).

The 16S rRNA gene sequences of six representative species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, *M. morgani*, *P. stuartii* and *P. rettgeri* were obtained and a phylogenetic tree constructed using the ClustalW alignment program (Thompson et al., 1994) and the tree-drawing program Phylodendron (Gilbert, D. G., 1989) to display the evolutionary relationships between the species in these genera. The tree (Fig. 1) indicates the close relationship between the two *Providencia* sp., as well as the similarity between two of the *Proteus* sp., *P. vulgaris* and *P. penneri*. The relationship between *P. mirabilis* and the *P. penneri*–*P. vulgaris* group is, however, more distant, a fact reflected in the horizontal distance between *P. mirabilis* and the other two *Proteus* sp. This greater distance at the 16S rRNA level between *P. mirabilis* and the other members of the genus *Proteus* may be a reflection of the phenotypic and physiological characteristics of *P. mirabilis*, which set it slightly apart from other members of the genus.

Electron micrographs of the six species, taken from overnight cultures grown in Luria-Bertani broth (LB; Ausubel et al., 1987), are shown in Fig. 2. Figure 2B is an electron micrograph of a *P. mirabilis* differentiated swarmer cell growing

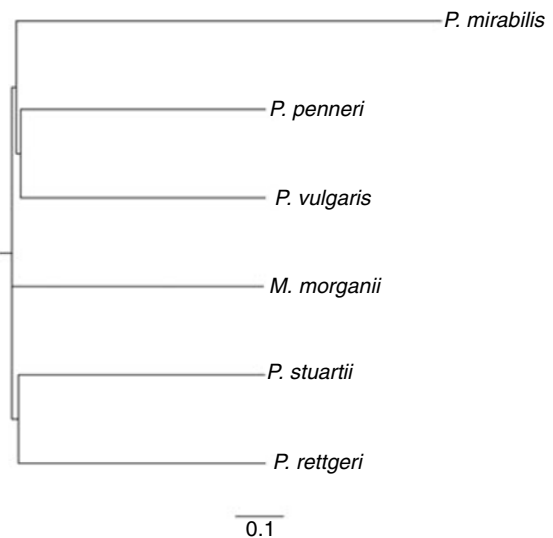
Phylogenetic tree of selected *Proteae*

Fig. 1. Unrooted phylogenetic tree of the 16S rRNA sequences from selected *Proteus*, *Providencia* and *Morganella* sp. The tree was derived using the ClustalW alignment program (Warren, J. W., 1987a) and the tree-drawing program Phylodendron (Fletcher, M. et al., 1994). The scale bar represents the expected number of changes per sequence position.

on Luria-Bertani agar (LBA). The cell elongation and hyperflagellation typical of this phenotype is clearly evident (see “Cell Differentiation and Swarming”).

## The Genus *Proteus*

### Habitat

*Proteus mirabilis* is the type species of the genus *Proteus* and by far the most extensively studied member of this genus. It was originally described and named by Hauser in 1885 for the Homer’s *Odyssey* character who “has the power of assum-



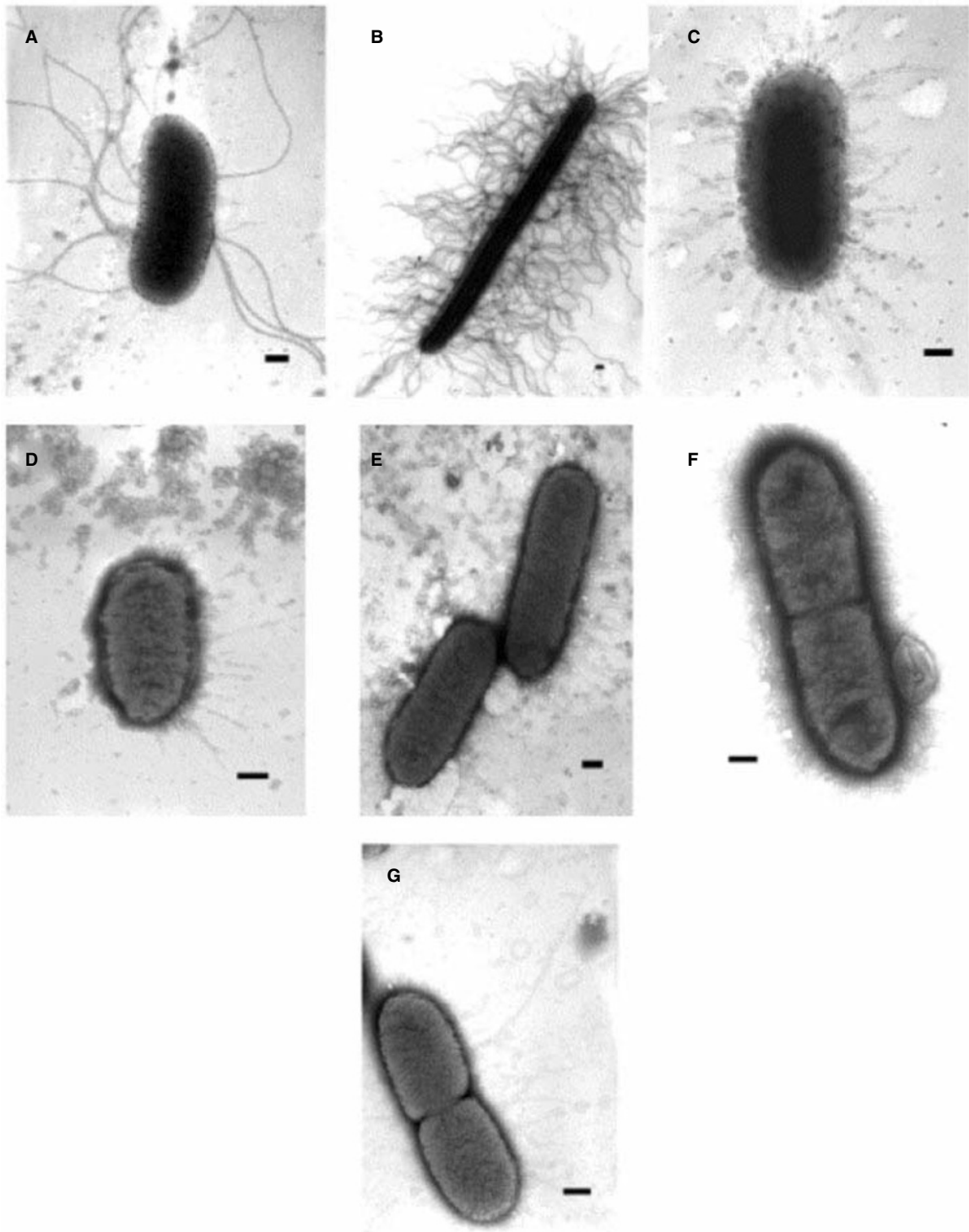


Fig. 2. Electron micrographs of selected species comprising the genera *Proteus*, *Morganella* and *Providencia*. (A) *Proteus mirabilis* swimmer cell; (B) *P. mirabilis* swarmer cell; (C) *P. vulgaris*; (D) *P. penneri*; (E) *M. morgani*; (F) *Providencia stuartii* and (G) *P. rettgeri*. The bar represents 200 nm.

ing different shapes in order to escape being questioned” (Hoeniger, 1964). Both *P. mirabilis* and *P. vulgaris* are widely distributed in the environment and have been isolated from the intes-

tinal tract of mammals, birds and reptiles. *Proteus mirabilis*, and to a lesser extent *P. vulgaris*, are common inhabitants of the human gastrointestinal tract. *P. mirabilis*, in particular, also

may colonize the urinary tract under certain circumstances, where it is considered an opportunistic pathogen and one of the principal causes of UTIs in hospital patients with indwelling urinary catheters.

*Proteus vulgaris* is also a common inhabitant of the human gut and a urinary tract pathogen; however, it is associated much less commonly with UTIs than *P. mirabilis*. For example, in a study of *Proteus* species found in urine from 217 hospital patients, Senior identified 258 strains of *P. mirabilis* compared to four strains of *P. vulgaris* (Senior, B. W., 1979).

*Proteus penneri* was first described as a species distinct from *P. vulgaris* in 1982 (Hickman et al., 1982). It has since been isolated from a number of diverse clinical sites, including abdominal wounds, urine samples, bladder calculi, epidural ulcers and bronchoalveolar lavage fluid (Krajden et al., 1984; Krajden et al., 1987; Latuszynski et al., 1998; Li et al., 1992).

The species *P. myxofaciens* has been isolated from both living and dead gypsy moth larvae (*Porthetia dispar*; Costas, M., et al., 1993). DNA/DNA hybridization studies and phenotypic similarity have formed the basis of its inclusion in the genus *Proteus* (Brenner et al., 1978). No further investigations have been reported on its characterization or pathogenicity in the host.

## Isolation and Identification

The species comprising the genus *Proteus* are distinguished biochemically from *Morganella* and *Providencia* spp. by their production of hydrogen sulfide and lipase, hydrolysis of gelatin and a lack of acid production from mannose (Table 2; Penner, J. L., and J. N. Hennessy, 1979b). Optimum growth conditions for these bacterial species are obtained at 37°C, which reflects the intestinal niche occupied by many of these bacteria. When grown in liquid media, *Proteus* sp. appears as short rods with six to ten peritrichous

flagella (Fig. 2A). Most strains also can differentiate into elongated hyperflagellated cells during growth on solid surfaces such as LBA (Fig. 2B), leading to the surface translocation event known as “swarming” (see “Physiology”). Swarming behavior makes it difficult to isolate single colonies for further study; however, colony isolation on agar can be obtained through an increase in the agar concentration to 20 g/liter and the addition of 5 ml glycerol per liter of medium. This has the effect of slowing down or preventing the initiation of swarming, leading to the formation of discrete colonies (Belas, 1992).

The spot indole test has been evaluated by Bale et al. (1985) as a rapid method of distinguishing *P. mirabilis* from *P. vulgaris* (Bale et al., 1985). In this evaluation, the majority (95.7%) of *P. mirabilis* strains gave a negative spot indole result. The predictive value was greater than 99%, if only isolates representing single strains were used, whereas *P. vulgaris* isolates were 88.9% positive by this method. Differential culture media also has been developed for presumptive screening of *Enterobacteriaceae*, which in turn can distinguish between genera in this family, including *Proteus* sp. (Hawkey et al., 1986a; Houang, E. T., et al., 1999; Manafi and Rotter, 1991).

## Physiology

CELL DIFFERENTIATION AND SWARMING. One significant phenotypic characteristic shared by members of the genus *Proteus* is the ability to transform into a distinctive “swarmer” cell when cultured on a solid agar-containing medium. Differentiation of *P. mirabilis* to the swarmer stage has been studied most extensively (Allison and Hughes, 1991; Belas, 1992; Williams and Schwarzhoff, 1978). When grown in liquid media, the cells exist as 1.5–2.0 µm rods with 6–10 peritrichous flagella. These so-called “swimmer” cells exhibit characteristic swimming and

Table 2. Distinguishing biochemical characteristics of the genera *Proteus*, *Morganella* and *Providencia*.

Biochemical Test	<i>Proteus</i>	<i>Morganella</i>	<i>Providencia</i>
Acid from mannose	–	+	+
Color on LIA	Red	Colorless	Red
Acid from inositol	–	–	+
Acid from D-mannitol	–	–	+
Acid from D-arabitol	–	–	+ <sup>a</sup>
Acid from adonitol	–	–	+ <sup>a</sup>
Acid from erythritol	–	–	+ <sup>b</sup>
Gelatin hydrolysis	+	–	–
Lipase production	+	–	–
H <sub>2</sub> S production	+	–	–

Symbols and Abbreviations: +, present; –, absent; and LIA, lysine iron agar.

<sup>a</sup>Negative for *P. stuartii*.

<sup>b</sup>Negative for 10–89% of *P. stuartii* strains.

chemotactic behavior, moving away from repellents and towards attractants (Allison et al., 1993; Lominski and Lendrum, 1947). Transfer of swimmer cells onto a solid growth medium, such as that containing agar, results in a remarkable physiological and morphological transformation of the bacteria. Shortly after contact with the surface, the swimmer cells begin to differentiate into a morphologically and biochemically unique cell known as “the swarmer cell” (Fig. 2B).

Swarmer cell differentiation and swarming behavior may be broken down into discrete steps. The first step in swarmer cell morphogenesis is cellular elongation, resulting from inhibition of the septation mechanism (Armitage et al., 1974). The molecular basis that underlies the inhibition of proper septum formation is not known, but may involve SulaA (also known as “SfiA”) or other proteins known in *Escherichia coli* to adversely affect septum formation (Higashitani et al., 1995; Huisman and D’Ari, 1981; Huisman et al., 1984). Belas et al. (1995) analyzed several *P. mirabilis* mutants defective in swarming and many of these strains had defects in genes encoding proteins necessary for cell wall structure. Elongated swarmer cells are typically 60–80  $\mu\text{m}$  in length and are polyploid, with the number of chromosomes per cell being roughly proportional to the increase in length. Concurrent with this, overexpression of the flagellin protein leads to the synthesis of hundreds to thousands of new flagella required for movement across the solid surface (Armitage and Smith, 1978; Hoeniger, 1965; Hoeniger, 1966; Houwink and van Iterson, 1950; Leifson et al., 1955). Diagrammatic representations of typical swimmer and swarmer cells are shown in Fig. 3, together with a summary of the main distinguishing features of the two cell types. These flagella are composed of the same flagellin subunit as the swimmer cell flagella, and in both cases flagellin is transcribed from the *flaA* gene, indicating that the same flagellar species is produced upon surface induction (Belas, 1994a; Belas and Flaherty, 1994b; Murphy and Belas, 1999).

Studies in *P. mirabilis* and in the swarming bacteria *Vibrio parahaemolyticus* and *Serratia marcescens* have shown swarmer-cell-specific genes are expressed when swimmer cells are transferred to solid media, suspended in highly viscous broths or agglutinated with antibody to the cell surface (Alberti and Harshey, 1990; Allison et al., 1993; Belas et al., 1986; McCarter et al., 1988; Stewart et al., 1997). All of these conditions result in inhibition of flagellar rotation, leading to the conclusion that the flagella act as tactile sensors of the external environment.

Swarmer cell differentiation and swarming behavior are inextricably linked, but a differentiated swarmer cell by itself is unable to swarm

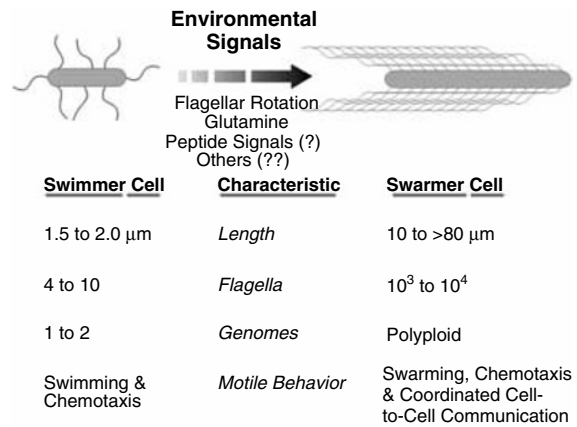


Fig. 3. The characteristics of *P. mirabilis* swarmer cell differentiation and swarming motility. Swarmer cell differentiation is controlled through a combination of sensing environmental conditions that reduce wild-type flagellar filament rotation and reacting to a specific chemical stimulus, the amino acid glutamine. The swarmer cell is characterized by an elongated polyploid cell that synthesizes numerous flagella in response to the aforementioned signals.

across a nutrient agar surface. Rather, swarming behavior is a cell-cell contact event that requires intimate contact and interaction between groups of swarmer cells to coordinate their movements (Bisset, 1973a; Bisset, 1973b; Bisset and Douglas, 1976; Brogan et al., 1971; Douglas and Bisset, 1976; Douglas, 1979). The arrangement of the coordinated swarmer cells during migration is illustrated in Fig. 4.

An important aspect of the *P. mirabilis* swarming colony pattern is its cyclic nature. As shown in Fig. 5, each cycle can be broken down into four parts, 1) swarmer cell differentiation, 2) the lag period prior to active movement, 3) swarming colony migration and 4) consolidation (where the cells stop moving and dedifferentiate back to swimmer cell morphology). During the migration phase, the fully differentiated swarmer cells move outward in unison in all directions from the original site of inoculation for a period of several hours. Movement then ceases and a process referred to as consolidation takes place (Bisset, 1973a; Bisset, 1973b; Hoeniger, 1964; Hoeniger, 1965; Hoeniger, 1966; Hoeniger and Cinitis, 1969; Williams and Schwarzhoff, 1978), during which the swarmer cells dedifferentiate back into swimmer cells. After a period in this stage, the swarming phase recommences and proceeds until the next consolidation phase. The cycle of swarming and consolidation is then repeated several times, until concentric rings, formed by the swarming bacteria and delineating the phase changes, cover the agar surface (Fig. 7). The purpose of the consolidation phase has yet to be fully elucidated; however, recent work by Matsuyama et

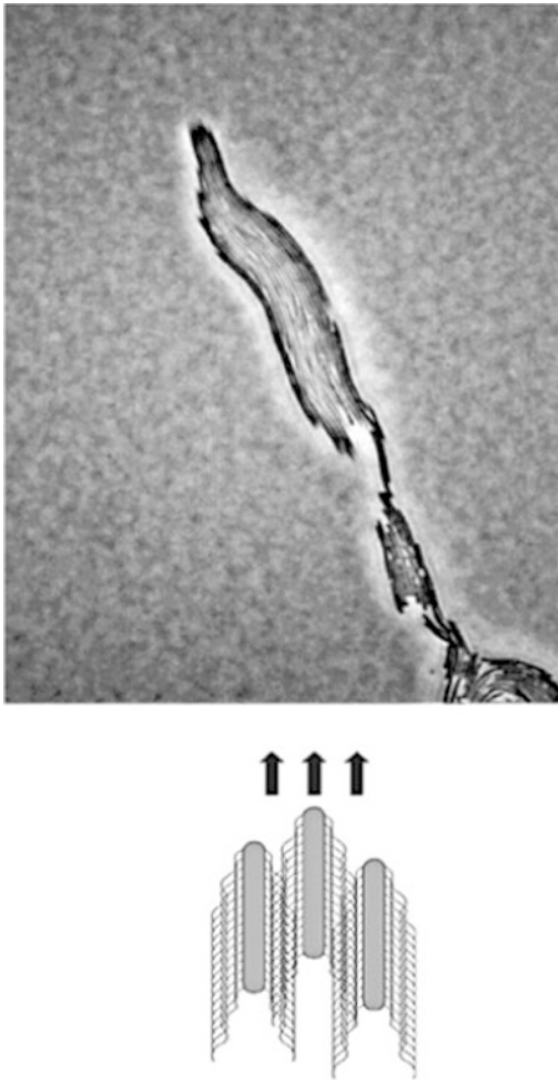


Fig. 4. The mechanics of swarming in *P. mirabilis* and the requirement for cell-cell contact. The micrograph (top) shows a finger-like projection of *P. mirabilis* swarmer cells migrating across a solid surface. The cartoon (bottom) demonstrates the arrangement of swarmer cells during coordinated motion across a solid surface. Individual cells are interlinked into groups and move en masse away from the point of inoculation, with the flagella from all cells in the group moving in unison. The arrows point in the direction of movement.

al. (2000) showed that the time course of this phase is unaffected by replica plating of the swarm edge. This indicates that consolidation is not a consequence of nutrient and metabolite changes in the medium. Figure 6 contains four video segments detailing the events associated with swarming at the microscopic level. Figure 7 shows swarming behavior during colony development and bulls-eye ring formation.

**THE INFLUENCE OF SIGNAL TRANSDUCTION IN SWARMING.** Swarming is, by

its very nature, a surface-associated and cell-density-dependent phenomenon. Individual *P. mirabilis* cells rely on their ability to sense the surrounding environment and use these cues to trigger the development of the swarmer cell, as well as to coordinate movement of the swarm across the surface. One way this information is acquired is through monitoring the rotation of the flagella to initiate cellular differentiation (see “Cell Differentiation and Swarming”). Other methods include cell-to-cell contact with neighboring bacteria to aid in movement, the chemotactic sensing of nutrients and repellents in the external medium, and possibly through a density-dependent sensing of cell population density known as “quorum sensing.” We will discuss each of these signal transduction mechanisms in turn in the next paragraphs.

Research into the relationship between chemotaxis and swarming in *P. mirabilis* (Belas et al., 1991a; Williams and Schwarzhoff, 1978) has preceded that into the relationship between chemotaxis and other swarming bacteria. Early work suggested that chemotaxis did not play a major role in the differentiation to the swarming phenotype (Williams et al., 1976). However, later studies demonstrated that nonswarming mutants of *P. mirabilis* produced by transposon insertion also exhibit deficiencies in chemotactic response (Belas et al., 1991b), suggesting that chemotactic signal transduction is important for swarming. Further work by Allison et al. (1993) has shown that the amino acid glutamine induces differentiation to the swarmer cell in *P. mirabilis* by acting as a chemoattractant. This effect may work at the level of transcription, because when glutamine is added to a defined, nonswarming medium, expression of the *flaA* (flagellin) and *hpmA* (hemolysin) genes in *P. mirabilis* increases 40-fold. The viscosity of the growth medium also affects swarming, with the addition of 3% v/v polyvinylpyrrolidone (PVP) to liquid chemotaxis medium resulting in increased attraction to glutamine. Attempts to repeat these experiments in other laboratories using different strains of *P. mirabilis* have been unsuccessful, raising doubts as to whether all *P. mirabilis* strains respond to glutamine by swarming. In other swarming members of the *Enterobacteriaceae*, Harshey and Matsuyama (1994) reported a link between chemotaxis and the swarming of *E. coli* and *Salmonella typhimurium* on specific (Eiken) agar.

Quorum sensing is the ability of a bacterial population to monitor its density through expression of small extracellular signaling molecules referred to as “autoinducers.” Quorum sensing was first identified and characterized in the luminescent marine bacterium *Vibrio fischeri* wherein bioluminescence is controlled by cell density and autoinducer signal transduction

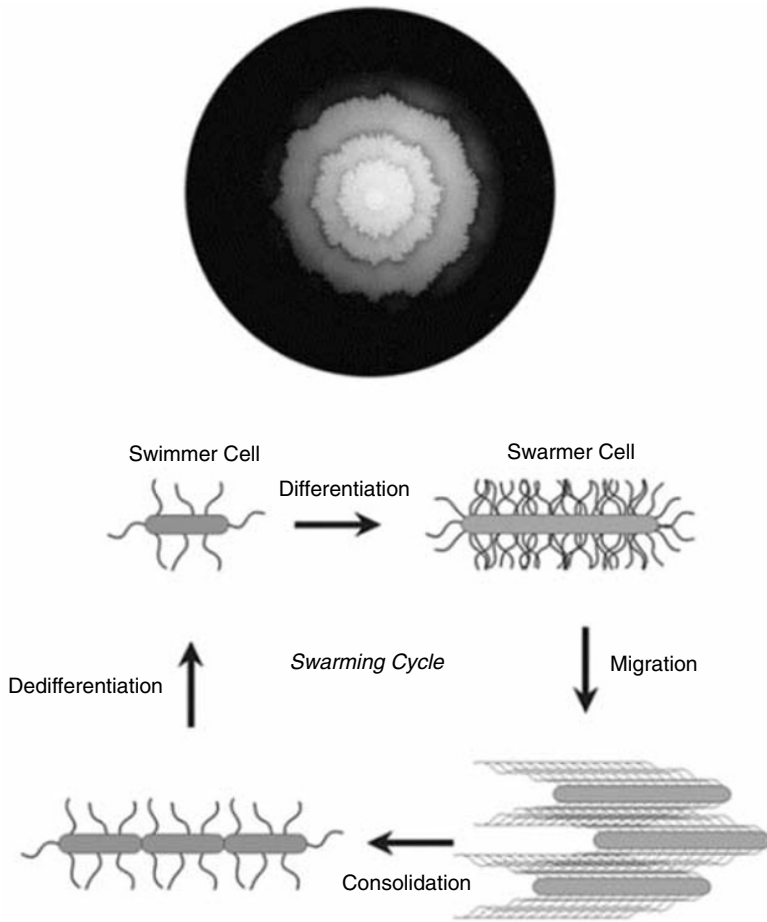


Fig. 5. The cyclic nature of *P. mirabilis* swarming behavior. Inoculation of *P. mirabilis* swimmer cells onto a solid nutrient surface such as Luria-agar induces the expression of swarmer-cell specific genes, leading to differentiation into the swarmer cell and migration away from the site of inoculation. This process is interspersed with periods of consolidation and dedifferentiation back into the swimmer cell morphology.

- (A) Pm 1 colony rt.asf
- (B) Pm 2 colony comp.asf
- (C) Pm 3 colony comp.asf
- (D) Pm 4 colony comp.asf

Fig. 6. Sequential events in the progress of the i) Cell Differentiation and Swarming phase of *P. mirabilis* differentiation. (A) A close up, in real time, of the periphery of a swarming colony. (B) Close up of colony development. (C) Swarming migration at the edges of a colony. (D) Close up of the periphery of the swarming edge in time lapse, showing cell-to-cell interaction. For the video, see the online version of *The Prokaryotes*.

(Eberhard, 1972; Eberhard et al., 1981; Neilson, 1977). The signal molecule, or autoinducer, is often a homoserine lactone with an *N*-acyl chain of variable length. In the case of *V. fischeri*, *N*-acylhomoserine lactone (AHL) can freely per-

meate the membrane and accumulate inside the cell until an optimum concentration is reached, whereupon it binds to a specific receptor and initiates activation of the bioluminescence genes (Kaplan and Greenberg, 1985). The specific receptors for the AHL signal are members of the LuxR family of transcription regulators. Two genes, *luxR* and *luxI*, were originally identified in *V. fischeri* as essential regulators of the AHL signaling system, and homologs of these have subsequently been identified in other Gram-negative bacteria (Stevens and Greenberg, 1998). In pathogenic bacteria such as *Pseudomonas aeruginosa*, AHL-mediated quorum sensing is involved in the regulation of multiple virulence determinants, including exoproteases, lipases and exotoxins, suggesting that a critical bacterial concentration must be attained for the deployment of its virulence factors (Parsek and Greenberg, 2000; Telford et al., 1998).

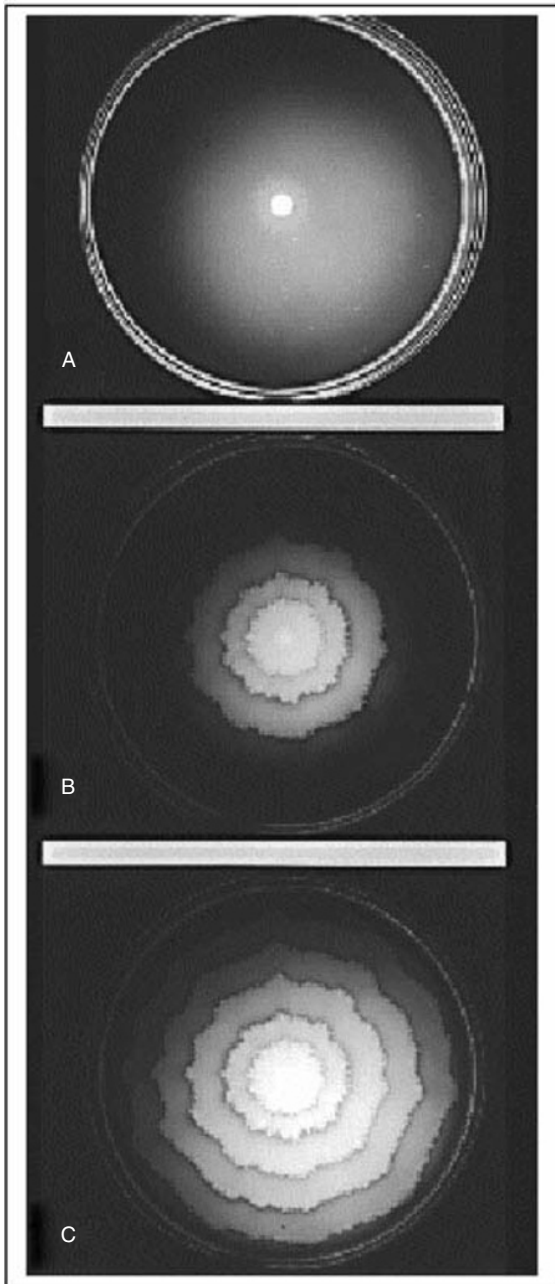


Fig. 7. Development of the bull's-eye rings of *P. mirabilis* colonies. (A–C) Sequential photographs of L-agar inoculated in the center with 5 ml of *P. mirabilis* swimmer cells and incubated at 30°C for 4 h (A), 24 h (B) and 48 h (C). (D) Video recording in time lapse showing swarming of *P. mirabilis* across the solid surface of an L-agar plate, taken over 24 hours of growth at 30°C and 80% humidity. For the video, see the online version of *The Prokaryotes*.

In *Serratia liquefaciens*, another swarming member of the *Enterobacteriaceae*, *S. liquefaciens*, Eberl et al. (1996) have demonstrated that initiation of swarmer-cell differentiation involves diffusible signal molecules that are released into the growth medium. In particular, the autoinducer *N*-acylhomoserine lactone (AHL) is required for swarming motility in this species

(Givskov et al., 1998; Lindum et al., 1998). The AHL derivatives *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone (HHL) were identified by Eberl et al. (1996) in cell-free *S. liquefaciens* culture supernatants.

The *swrI* (swarmer initiation) gene, whose predicted translation product exhibits substantial homology to the LuxI family of putative AHL synthases, is responsible for directing synthesis of both BHL and HHL (Eberl et al., 1996). Further work by this group since has shown that the coordinate expression of *swrI* and the flagellar master operon *flhDC* is required to initiate swarming motility in *S. liquefaciens* (Eberl et al., 1999; Givskov et al., 1998; Lindum et al., 1998).

Attempts to prove the existence of an AHL-type autoinducer in *P. mirabilis* have been unsuccessful; however, evidence exists that this bacterium may utilize an alternate method of density-dependent quorum sensing. Belas et al. (1998) have characterized a gene that upon mutation decreases the length of the lag phase prior to swarming. This gene, referred to as “*rsbA*” (for regulator of swarming behavior) encodes a sensory protein displaying similarity to LuxQ and other bacterial histidine kinases of the two-component regulatory superfamily of bacterial response regulators that perform functions required for a second density-sensing system (Bassler et al., 1994; Freeman and Bassler, 1999; Lilley and Bassler, 2000). This finding suggests that RsbA may function as a sensor of environmental conditions required to initiate swarming.

## Ecology

Both *P. mirabilis* and *P. vulgaris* are members of the normal flora of the mammalian intestinal tract and have been isolated from humans, dogs, monkeys, pigs, sheep, cattle, raccoons, cats, rats and other mammals. They also are distributed widely in the environment, with reservoirs in soil, water, sewage and feces (Guentzel, 1991). Other species of *Proteus* are less widely distributed. For example, *P. penneri* is absent from the intestines of livestock (Hawkey et al., 1986b), whereas *P. myxofaciens* is confined to the larvae of the gypsy moth (Cosenza and Podgwaite, 1966).

## Epidemiology

Owing to their varied habitats, members of the genus *Proteus* and related genera have many possible routes of human infection. The modes of transmission may include nosocomial sources, such as hospital food and equipment, intravenous solutions and human contact through contaminated skin surfaces. Long-term indwelling catheters are a prime site of colonization and

infection for *P. mirabilis* and *P. vulgaris* (Kunin, 1989; Stickler and Hughes, 1999; Warren, 1987a).

Serological typing of *P. mirabilis* and *P. vulgaris* traditionally has been done using the slide agglutination test and the indirect hemagglutination test (Gmeiner et al., 1977; Schmidt et al., 1970). Previously, the Dienes test, in which swarming *Proteus* strains were inoculated on nutrient agar and allowed to swarm into one another, was used to distinguish between strains of these species. If a distinct line of demarcation (a Dienes line) occurred at the junction between the strains, they were considered different (Dienes, 1946). This method has fallen out of favor because of the difficulties associated with interpreting the results.

Other bacteriological typing methods also have been used, including biotyping, bacteriophage typing, and typing schemes using both bacteriocin production and bacteriocin sensitivity (Anderson and Engley, 1978; Hickman and Farmer, 1976; Kusek and Herman, 1980; Schmidt and Jeffries, 1974). In a comparative study by Kusek (1981), five bacteriological typing methods were assessed for their ability to differentiate 100 clinical isolates of *P. mirabilis*. The highest sensitivity and specificity was obtained using bacteriocin production typing, which yielded 29 distinct bacteriocin types among the 80% of strains that were typable (Kusek and Herman, 1981). Bacteriophage typing sets also have been tested as tools for differentiation of *Proteus* strains; however, a more diverse set of phages is needed to adequately differentiate between as many strains as possible (Sekaninova et al., 1994; Sekaninova et al., 1998).

Modern molecular methods employing the polymerase chain reaction (PCR) to produce DNA fingerprints and other 16S ribosomal RNA gene (ribotyping) methods of strain differentiation have been applied to distinguish *P. mirabilis*, *P. vulgaris* and *P. penneri* strains (Costas et al., 1993; Hoffmann et al., 1997; Hoffmann et al., 1998; Serwecinska et al., 1998). Hoffmann et al. (1998) compared four typing methods (including plasmid profiles, outer-membrane-protein profiles, randomly amplified polymorphic DNA PCR [RAPD-PCR], and restriction fragment length polymorphism [RFLP]) on strains of *P. penneri* and found that RAPD-PCR alone, with one of two random primers, revealed 13 reproducible typeable patterns (Hoffmann et al., 1998). Thus, the RAPD method, essentially a DNA fingerprinting method using arbitrary primers (Akopyanz et al., 1992), revealed a significant DNA diversity among *P. penneri* strains that was not detected by other methods (Hoffmann et al., 1997; Hoffmann et al., 1998). The RAPD-PCR technique has the advantage of being quick and economical, with high reproducibility and typability; however, the choice of primers is critical to

obtaining discriminating results (Madico et al., 1995).

### Pathogenicity

The incidence of UTI involving *P. mirabilis* or *P. vulgaris* is lower than that for *E. coli*; however, *P. mirabilis* infections are more likely to be long-term and persistent, and to lead to greater complications threatening the patient's health than those involving *E. coli* (Mobley and Warren, 1987). The majority of *Proteus* infections are associated with prolonged hospitalization and the complications of long-term urinary catheterization. The complications that can result from *Proteus* UTI include catheter and urinary tract obstruction, kidney stone formation (urolithiasis), pyelonephritis, fevers and bacteremia (Mobley and Warren, 1987; Rubin et al., 1986; Senior, 1983; Story, 1954; Warren et al., 1987b). In serious cases of *Proteus* UTI, chronic renal inflammation, vascoureteral reflux and renal failure are frequently observed (Cohen and Preminger, 1996; du Toit et al., 1995; Warren, 1987a). The sites of UTI and the respective complications resulting from infection at these sites are shown in Fig. 8, whereas Fig. 9 provides a breakdown of the causes of *P. mirabilis* UTI.

**VIRULENCE FACTORS.** At least ten virulence factors potentially contributing to the pathogenicity of *Proteus* sp. have been identified. The majority of these virulence factors, with the exception of the fimbriae, are expressed during swarmer cell differentiation and swarming behavior. These virulence factors can be divided into two groups: 1) proteins, enzymes and other secreted products, and 2) surface structures. In terms of their relevance to pathogenesis, the most significant virulence factors (in order of importance) are: urease, ZapA (a protease that specifically degrades immunoglobulins IgA and IgG), lipopolysaccharide, outer-membrane proteins, and hemolysin. The most significant surface structures, in order of importance to pathogenesis, are the flagella and associated swarming phenotype of *P. mirabilis* and *P. vulgaris*, and several types of fimbriae, including; mannose-resistant *Proteus*-like fimbriae (MR/P), mannose-resistant *Klebsiella*-like fimbriae (MR/K), *P. mirabilis* fimbriae (PMF), nonagglutinating fimbriae (NAF), and an uncharacterized fimbrial type with a 24-kDa major subunit (also referred to as "F24"). The potential of these virulence factors in *Proteus* to cause disease has been a prime area of study and the major conclusions in each case are presented here.

**Urease.** Urea is a by-product of nitrogen metabolism that is ubiquitous in a wide range of eukaryotes and prokaryotes. The enzyme urease breaks down urea to ammonia and carbon dioxide. These end products have the effect of

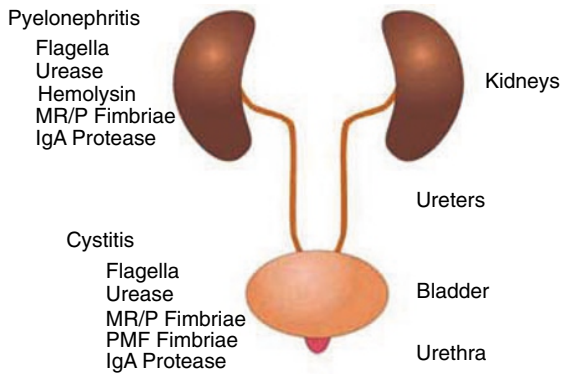


Fig. 8. The human urinary tract, showing the sites of *P. mirabilis* infections, hence the diseases resulting from infection, and the virulence factors associated with pathogenesis of disease at the respective sites (see “Pathogenicity”).

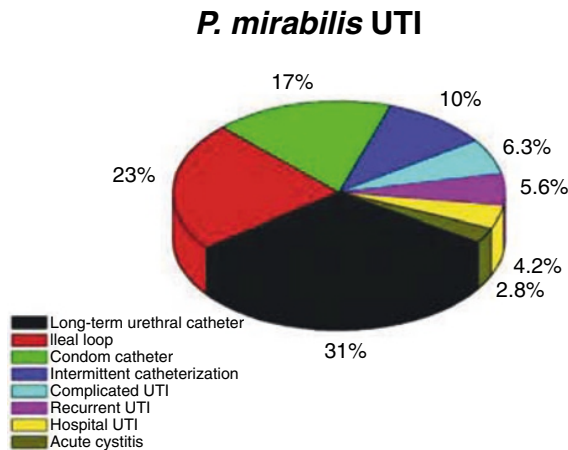


Fig. 9. The causes of *P. mirabilis* urinary tract infections (UTI) in humans, compiled using epidemiological data from hospitalized patients. Adapted from Mobley and Warren (1987).

increasing the pH, which in turn augments the survival of bacteria such as *Helicobacter pylori* to create a less acidic environment that may be more suited for colonization (Gomez-Duarte et al., 1998; Kuwahara et al., 2000; Lee et al., 1999). In other microbes, ureases play an important role in utilization of environmental nitrogenous compounds and urea-based fertilizers. At the same time, the production of urease by *P. mirabilis* and *P. vulgaris* may contribute to the development of urinary stones and pyelonephritis, as mentioned earlier. This is one possible reason why *Proteus* infections cause more cytological damage than *E. coli* infections (Cohen and Preminger, 1996; du Toit et al., 1995; Johnson et al., 1993; Mobley and Hausinger, 1989).

Early studies using *P. mirabilis* treated with urease inhibitors established a link between urease and colonization of rat urinary tracts. In

these studies, the renal tissue of control-infected rats contained a far higher number of bacteria and had greater tissue damage than did those of rats infected with inhibitor-treated *P. mirabilis* (Musher et al., 1975). Further evidence of the role played by urease in *P. mirabilis* pathogenesis has been demonstrated by comparative histological examination of renal tissues postinfection by either the wild type, parental strain, or an isogenic urease-negative mutant using a mouse model of ascending UTI (Johnson et al., 1993; Jones et al., 1990). Mice challenged with the isogenic urease mutant developed significantly less bacteriuria and urinary stones compared to the parent strain. The parent strain also showed greater persistence in the bladder and kidney than did the strain lacking urease (for reference to sites of infection, see Fig. 8).

**IgA and IgG Proteases.** Secretory immunoglobulins of the IgA class are produced by mucous tissue and are particularly resistant to enzymatic breakdown by proteases. The ability to degrade a host's secretory IgA may provide a microorganism with an advantage by evading the host immune response, thus gaining valuable time for the bacterium to establish a foothold for colonization. Many pathogenic bacteria that invade mucosal tissues, such as the epithelial lining of the intestine and urogenital tract, have potent, extracellular IgA-degrading proteases, whereas nonpathogenic counterpart species in the same genus often do not. For example, pathogenic species of *Neisseria*, such as *N. gonorrhoeae* and *N. meningitidis*, possess IgA-degrading proteases, though nonpathogenic species (including *N. lactamica* and *N. sicca*) do not (Kilian et al., 1983). The possession of IgA protease by certain members of this genus may thus be critical in providing these bacteria with an advantage to overcome the host humoral immune response during infection.

A protease capable of degrading two IgA subclasses (IgA1 and IgA2) as well as IgG has been identified in *P. mirabilis* (Loomes et al., 1990; Milazzo and Delisle, 1984; Senior et al., 1987b). Subsequent work by Wassif et al. (1995) has resulted in the characterization of this protease (referred to as “ZapA” and encoded by the *zapA* gene) as a metalloprotease of ca. 50 kDa. To investigate whether *zapA* expression correlates with another known virulence-enhancing phenomenon, swarmer-cell differentiation (see “Flagella and swarming”), Walker et al. (1999) measured the expression of *zapA* during swarmer-cell differentiation. The data obtained suggest that *zapA* expression is tightly coordinated not just with swarmer-cell differentiation, but with swarming behavior as well. Also, ZapA proteolysis is not essential for swarming, because ZapA<sup>-</sup> strains were shown to produce wild-type swarmer cells and swarming colonies.



Both *P. vulgaris* and *P. penneri* also have been reported to possess IgA proteases. In a survey of protease production amongst 24 *P. vulgaris* strains, Senior et al. (1988) found that half of them produced IgA protease. Each of five of the *P. penneri* strains surveyed in the same study also produced IgA protease. Subsequent purification and comparative analysis of IgA protease from *P. mirabilis*, *P. vulgaris* and *P. penneri* showed that they shared similar electrophoretic patterns on sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, with only slight differences in protein band mobility (Loomes et al., 1992).

**Lipopolysaccharide and Outer-Membrane Proteins.** During infection, bacterial lipopolysaccharide (LPS) is a primary target antigen (O-antigen) for host immunologic responses. Bacterial LPS activates macrophages to produce toxic oxygen radicals, interleukin-1 and tumor necrosis factor, leading to a wide range of effects in the host, including hypertension, fever and lethal shock (Hamilton and Adams, 1987; Rietschel et al., 1994). By way of example, the effect of *P. mirabilis* LPS on pig blood platelets is akin to that of thrombin (a strong platelet agonist), stimulating the enzymatic cascade leading to platelet aggregation (Wachowicz et al., 1998).

The outer membrane of Gram-negative bacteria contains several major proteins that are complexed with other membrane components such as LPS and phospholipid. The polysaccharide chains of the different species of *Proteus* LPS contain sufficient structural differences to result in an antigenically heterogeneous genus. These differences have been used to cluster *P. mirabilis*, *P. vulgaris*, and *P. penneri* strains into serogroups based on their agglutination when mixed with antibodies directed against specific species of LPS molecules (Kotelko, 1986; Penner and Hennessy, 1980a; Perch, 1948; Zych and Sidorczyk, 1989). Certain LPS epitopes have been investigated to determine their role in antigenic specificity. The particular groups on the oligosaccharides found to play a dominant role in the specificity of *P. mirabilis* and *P. penneri* LPS are the amide of D-galacturonic acid with L-lysine  $\alpha$ -D-GalA-(L-Lys) (and the amide of D-galacturonic acid with L-threonine  $\alpha$ -D-GalA-[L-Thr]), respectively (Radziejewska-Lebrecht et al., 1995; Sidorczyk et al., 1995).

Specific *P. mirabilis* outer-membrane proteins (OMPs) have been the targets of study with respect to their effects on the host immune response. One particular 39-kDa major OMP of *P. mirabilis* (subsequently identified as "OmpA") has been shown to greatly enhance the host IgG response for LPS in mice when complexed to LPS (Karch and Nixdorff, 1981; Karch and Nixdorff, 1983). Also, LPS is an activator of mac-

rophages, another important component of the host immune response, through their production of toxic oxygen radicals, interleukin-1 and tumor necrosis factor (Guthrie et al., 1984). Weber et al. have shown that OmpA inhibits the LPS-induced oxidative response and interleukin-1 production of murine macrophages when complexed to LPS, thus acting as a modulator of the interaction of LPS with macrophages (Weber et al., 1992; Weber et al., 1993).

**Hemolysin.** Bacterial hemolysins are frequently involved in the destruction of erythrocytes during infection and have been shown to contribute to the invasiveness and pathogenicity of several bacterial species (Braun and Focareta, 1991; Braun et al., 1993; Goebel et al., 1988; Menestrina et al., 1995). The importance of hemolysin in the virulence of *E. coli* was demonstrated through the isolation, cloning and transfer of hemolysin genes from virulent strains to avirulent ones (Hacker et al., 1983; Welch et al., 1981).

The association between hemolysin and virulence in *Proteus* sp. has proved more difficult to establish. Hemolytic activity is the least significant virulence factor in strains of *P. mirabilis* recovered from the CBA mouse model of ascending UTI (Fig. 10). However, early work by Peerbooms et al. (1985) showed a correlation between the hemolytic activity of *P. mirabilis* and its virulence in mice (Peerbooms et al., 1983). Hemolytic strains of *P. mirabilis*, *P. vulgaris* and *P. penneri* demonstrated markedly greater invasiveness of human and sheep erythrocytes in

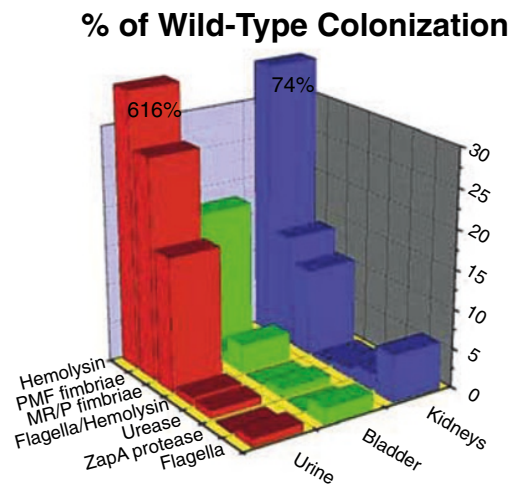


Fig. 10. The major virulence factors of *P. mirabilis* and their influence on urinary tract pathogenesis using the CBA mouse model of ascending UTI. Data are represented as percent survival of each isogenic mutant strain compare to the survival of wild-type parental strain postinfection in the CBA mouse model of UTI. Adapted from Mobley and Warren (1987).

vitro in work by Rozalski and colleagues (Rozalski et al., 1986; Rozalski and Kotelko, 1987).

At the molecular level, the hemolysin structural genes (*hlyA* and *hlyC*) and the principal secretion gene (*hlyB*) of *E. coli* can be functionally complemented by the homologous genes from *P. mirabilis*, *P. vulgaris* and *M. morganii*, suggesting a functional relatedness between the three components in the two species. A second *E. coli* secretory gene (*hlyD*) is present in *P. vulgaris* and *M. morganii*, but absent from *P. mirabilis* (Koronakis et al., 1987; Koronakis and Hughes, 1988a; Koronakis et al., 1988b). The *E. coli* *hlyD* gene is required for secretion of hemolysin (Pimenta et al., 1999). This finding may explain why the secreted hemolytic activity of *P. mirabilis* remains cell-associated rather than being released into the surrounding medium. This possibility has been supported by subsequent investigations showing that the secreted hemolysin (HpmA) of *P. mirabilis* and *P. vulgaris* is genetically distinct from that of *E. coli* (Swihart and Welch, 1990b; Welch, 1987). *Proteus mirabilis* HpmA is cytotoxic for a variety of cell lines, including cultured human renal proximal tubular epithelial cells (Mobley et al., 1991; Swihart and Welch, 1990a). As mentioned above, colonization by isolates showing hemolysin activity forms by far the greatest proportion of *P. mirabilis* isolates recovered from the urine, kidneys and bladder of CBA mice with UTI (Fig. 10; Mohr O'Hara, C., et al., 1999). However, HpmA activity in such clinical isolates does not appear to be crucial to the development of pathogenesis (Mobley and Chippendale, 1990). Thus, hemolysin is not considered as important a virulence factor in *P. mirabilis* pathogenesis as flagella or urease (Mobley and Belas, 1995). In the case of *P. vulgaris*, hemolysin production, measured as hemolytic titer (Peerbooms et al., 1983), was found by Peerbooms et al. (1985) to be significantly lower than that for *P. mirabilis*, thus reducing the relevance of hemolysin in UTI for this species.

**STRUCTURES INVOLVED IN THE VIRULENCE OF PROTEUS SP. Flagella and Swarming.** It is generally accepted that flagella and swarming behavior play some role in the pathogenicity of *P. mirabilis*, and several studies support this hypothesis. For example, in work by Allison et al. (1992) using two different human uroepithelial cell lines (EJ/28 and 5637) and *P. mirabilis* mutants lacking flagella (*Fla*<sup>-</sup>), the loss of flagella resulted in a noninvasive bacterium when compared to the wild-type parent. In the same study, the ability to invade the EJ/28 and 5637 cell lines was closely identified with differentiated swarmer cells, which were 15-fold more invasive than undifferentiated swimmer cells. In *in vivo* studies using a mouse model of UTI, Mobley et

al. (1996) reported a 100-fold lower recovery of a *Fla*<sup>-</sup> mutant defective in flagellar filament synthesis when compared to the parent strain. While nonflagellated strains are less invasive, flagella do not appear to be absolutely required for virulence. Zunino et al. (1994) have found that a naturally occurring nonmotile strain could infect mice and cause UTI, whereas Legnani-Fajardo et al. (1996) reported similar results with an isogenic mutant lacking flagella.

The differentiated swarmer cell (see "Physiology") probably provides *P. mirabilis* and other swarming species such as *V. parahaemolyticus* with the advantage of rapid colonization during pathogenesis. Apart from the benefits of motility and adherence due to hyperflagellation, swarmer cell differentiation in *P. mirabilis* coincides with increased expression of several virulence factors (see "Virulence Factors") and has been linked to the expression of extracellular (capsular) polysaccharide. Evidence for this has been provided in studies by Gygi et al. (1995) utilizing transposon mutagenesis of a gene coding for a cell surface (capsular) polysaccharide in *P. mirabilis*. The mutants clearly showed retardation in translocation velocity across solid media and were attenuated in their ability to establish UTI in mice (Allison et al., 1994; Gygi et al., 1995).

**Fimbriae.** Collectively, the different species of fimbriae, which can be distinguished from flagellae by their shorter "spiked" appearance (Fig. 2C) are important in the attachment of the bacteria to the epithelial cell surfaces and colonization of the surrounding tissues (Silverblatt, 1974). Fimbriae appear to be differentially expressed at particular stages of the swarming cycle (Fig. 5) and are absent on hyperflagellated swarmer cells (Hoeniger, 1965; Latta et al., 1999). *Proteus mirabilis* produces MR/P, MR/K, NAF and PMF fimbriae (Adegbola et al., 1983; Bahrani et al., 1993a; Bahrani et al., 1993b). Both MR/P and MR/K fimbriae also have been shown to function as hemagglutinins and are highly immunogenic.

Mutants defective in the synthesis of various fimbrial species have been used to assess the role of each fimbrial type during *P. mirabilis* UTI. In particular, MR/P mutants (*MrpA*<sup>-</sup>) have been constructed by allelic exchange and are between 6 and 28-fold less efficient in colonizing mouse urinary tracts than the parental strain of *P. mirabilis* (see also Fig. 10). This result is supported by histopathology results showing less damage to the uroepithelium and no signs of pyelonephritis during colonization by *Mrp*<sup>-</sup> strains (Bahrani et al., 1994). In studies on PMF fimbriae, isogenic *PmfA*<sup>-</sup> mutants were found to colonize the bladder at lower levels than the wild-type parent, but bacterial colonization of the kidney was not affected by loss of this fimbrial type (Massad et al., 1994). The involvement of NAF fimbriae in bacterial adherence also has been demonstrated

in *P. mirabilis* strains that express NAF as their only fimbrial species. These studies have shown that NAF provide strong adherence to a number of mammalian cell lines in vitro, including uroepithelial cells (Latta et al., 1998; Tolson et al., 1995; Tolson et al., 1997). While it would appear that the production of fimbriae offers the bacterium a distinct advantage for survival in colonizing host tissues, one of the disadvantages of fimbriae to the potential colonizer is that they render the bacteria more susceptible to phagocytosis (Silverblatt and Ofek, 1978). Furthermore, the presence of fimbriae does not equally enhance adherence to all cell types. For example, the use of MR/P fimbrial mutants (*mrpA*<sup>-</sup>) in the CBA mouse model of ascending UTI has shown that the loss of MR/P fimbrial expression does not completely prevent colonization of renal tissue in *P. mirabilis* (Bahrani et al., 1994).

## The Genus *Morganella*

### Habitat

The sole species in this genus, *Morganella morganii*, is a commensal organism that can rapidly colonize the host gut with an accompanying hypertrophy of Peyer's patches and development of specific IgA responses in the lamina propria cells (Shroff et al., 1995). Strains of *M. morganii* also are known to infect the human urinary tract, respiratory system and blood, though they have only been recovered occasionally from these sources (Braunstein and Tomasulo, 1978).

### Isolation and Identification

Isolation of *M. morganii* is accomplished using media for the routine isolation of *Enterobacteriaceae*. Enrichment media for culturing *M. morganii* from fecal specimens frequently include tetrathionate and selenite, which are added to nutrient broth (Rustigian and Stuart, 1945). The biochemical tests used to identify and distinguish *M. morganii* are shown in Table 2. It should be noted that although *M. morganii* is urease and indole positive, it does not swarm and is negative for most of the biochemical reactions characteristic of the *Proteus* spp.

The isolation of atypical *M. morganii* strains whose characteristics differ from those listed in Table 2 has resulted in the creation of a number of distinct biogroups that subdivide the species. Hickman et al. (1980) described 19 strains that were lysine positive and fermented glycerol within 24 h, in contrast to the type strain of *M. morganii*, which is lysine negative and ferments glycerol slowly or not at all. Another group of 14 *M. morganii* strains was found to be ornithine negative, whereas the type strain is ornithine positive. Because both groups were closely

related to *M. morganii* by DNA-DNA hybridization, they were considered distinct biogroups. There are currently seven recognized biogroups, based on ornithine and lysine decarboxylase reactions; however, some strains may carry plasmid-borne genes that code for these enzymes (Cornelis et al., 1981). Other phenotypes also have been used to distinguish the various biotypes. Janda et al. (1996) found resistance to the antibiotic tetracycline to be a useful distinguishing characteristic in their classification of 73 strains of *M. morganii* principally recovered from routine clinical specimens. The future subdivision of *M. morganii* into two or more species based on differences between biotypes remains a distinct possibility as more characteristics are used to distinguish between the biotypes.

### Ecology

*Morganella morganii* occurs in low numbers in the feces of healthy humans and animals, including dogs, cattle and chickens (Hawkey et al., 1986b; Phillips, 1955; Prasad and Pandey, 1966; Tanaka et al., 1995). Its habitat may be more far-reaching, as *M. morganii* strains have been isolated from snakes, chickens suffering from respiratory disease, and ocular lesions of harbor seals and elephant seals (Lin et al., 1993; Muller, 1972; Thornton et al., 1998). It is unclear whether *M. morganii* was the causative agent in these diseases or an opportunistic colonizer of the previously diseased tissue.

### Epidemiology

The incidence of *M. morganii* in diarrhea has been studied by Muller (1986a). In this study of fecal specimens from diarrheal and non-diarrheal patients, Muller isolated *P. mirabilis* and *M. morganii* more frequently from human diarrheal cases than from the stools of healthy individuals. These results agree with the data from earlier studies that showed a similar pattern of *M. morganii* recovery from diarrheal patients (Ahren, 1990; Das, 1996).

Epidemiological typing schemes have been developed for *M. morganii* based on the bacterial somatic and flagellar (O and H, respectively) antigens (Penner and Hennessy, 1979b; 1979d; Rauss and Voros, 1959; Rauss and Voros, 1967a; Rauss and Voros, 1967b; Rauss et al., 1975). The typing of *M. morganii* strains using the lytic activity of bacteriophages has been investigated in detail by Schmidt and Jeffries (1974). Seven phages were isolated from three *M. morganii* strains and these phages were successfully used to differentiate 13 of the 19 *M. morganii* strains in the study. Furthermore, lytic patterns remained stable in randomly selected *M. morganii* isolates retested several weeks later. While

phage typing is no longer widely used in the United States, these methods are still in favor in many East European countries.

A bacteriocin (referred to as “morganocin”) typing scheme for *M. morganii* based on production of and sensitivity to the protein has been described by Senior (Senior, 1987a). A total of 160 *M. morganii* strains were tested for sensitivity to morganocin and classified according to morganocin production and sensitivity. Most strains (97.5%) were sensitive to several different types of morganocins. Subsequent typing studies found that a combination of three distinct methods (bacteriocin typing, O-antigen serotyping and protein profiling) could be used to achieve a much greater degree of strain discrimination, especially inasmuch as protein profiling appears to be independent of O-serotype and bacteriocin type (Senior and Voros, 1989; Senior and Voros, 1990).

### Pathogenicity

While the etiological role of *M. morganii* in diarrhea has not yet been firmly established, it is consistently recovered from the feces of diarrheal patients suggesting an involvement in the disease. Furthermore, some researchers have found *M. morganii* to be the sole potentially pathogenic bacterial species in the feces of diarrheal patients, thus strengthening its claim to being the cause of the disease in these cases (Rauss, 1936; Senior and Leslie, 1986).

In spite of the involvement of *M. morganii* in diarrheal disease, this species is less likely to be the causative agent of human UTI than *P. mirabilis*. This is mainly due to the slower growth rate of *M. morganii* in urine compared to that of *P. mirabilis* and the noninducible nature of its urease (Senior, 1983). Although not a major contributor to human UTI, *M. morganii* has been implicated in outbreaks of septicemia and bacteremia in humans and animals (Bagel and Grossman, 1985; Barragan Casas et al., 1999; Heard et al., 1988; McDermott and Mylotte, 1984; Novak and Seigel, 1986; Rowen and Lopez, 1998). *Morganella morganii* bacteremia most commonly occurs in postoperative patients who receive  $\beta$ -lactam antibiotics. McDermott and Mylotte (1984) investigated the case histories of 19 documented episodes of *M. morganii* bacteremia in 18 hospital patients and showed that the majority of infections were either postoperative or had associated wound injuries. They concluded that *M. morganii* is an infrequent cause of bacteremia and its presence in blood cultures may be an indicator of an environment, such as surgery, that is conducive for an outbreak of nosocomial infection.

**VIRULENCE FACTORS.** There is very little known about the virulence factors involved in *M.*

*morganii* pathogenesis. Despite the paucity of reports, the most significant of these virulence factors are described below, though it should be noted that the data supporting the efficacy of each virulence factor may be minimal or not as substantial as those for the virulence factors of *P. mirabilis*, for example.

**Hemolysin.** The synthesis of active intracellular hemolysin (Hly) by *M. morganii* follows a pattern similar to that seen with the hemolysins from *E. coli*, *P. mirabilis* and *P. vulgaris*. Emody et al. (1980) measured the virulence of *M. morganii* strains due to hemolysin. Hemolysin (Hly<sup>+</sup>) wild-type strains and Hly<sup>+</sup> transconjugants were found to be more virulent than Hly<sup>-</sup> in mice and chick embryos. This enhanced virulence seems to be connected with the production of a diffusible  $\alpha$ -hemolysin. There is an important difference between the hemolysins of *M. morganii* and those of *P. mirabilis* and *P. vulgaris*. Koronakis et al. (1987) found that hemolytic activity in all *M. morganii* strains tested was cell free (extracellular), whereas in all *P. mirabilis* and 60% of *P. vulgaris* strains, hemolysin was only found associated with intact cells. The presence of the secretory gene *hlyD* in *M. morganii*, may explain the difference in secretion patterns observed in these two genera.

**Urease.** While both *P. mirabilis* and *M. morganii* possess urease activity with some similarities, there are also significant differences between the enzymes that may play a role in choice of habitat and pathogenicity. Both species possess ureases, there are distinct differences between the enzymes in the two species that may play a role in habitat and pathogenicity. For example, *M. morganii* urease possesses a higher affinity for urea than *P. mirabilis* urease, but the latter hydrolyzes urea at a rate 6- to 25-fold faster (Jones and Mobley, 1987). *Morganella morganii* survives in acidic conditions and its ureases have been shown to be activated in vitro by low pH, with an unusually low activity optimum of pH 5.5 (Young et al., 1996). In this respect, the urease from *M. morganii* is similar to the urease of *Y. enterocolitica* and *H. pylori*, both of which can hydrolyze urea at significantly higher rates under acidic conditions when compared with other pathogenic bacteria. A critical assessment of whether the *M. morganii* urease is a significant factor in the pathogenesis of this species will ultimately require in vivo studies with urease-negative mutants.

## The Genus *Providencia*

### Habitat

The members of the genus *Providencia* are all facultative anaerobes and motile by peritrichous flagella (Figs. 2F, G); however, they do not

exhibit cellular differentiation and swarming behavior. Urease production is not characteristic of all *Providencia* species, with only *P. rettgeri* strains producing urease (Brenner et al., 1978).

*Providencia stuartii* is found most frequently in hospital patients with urinary tract infections. Less frequently, it is found in respiratory and skin infections (Stickler et al., 1985; Warren, 1986). *Providencia alcalifaciens* is generally isolated from stool samples along with other enteric pathogens. A similar species (*P. rustigianii*), was originally isolated from human feces as *P. alcalifaciens* biogroup 3 (Ewing et al., 1972) and subsequently redesignated as a separate species (Hickman-Brenner et al., 1983). It has since been isolated from a range of human and animal sources, including (oddly enough) penguin feces (Costas et al., 1987; Muller, 1983). While this species can colonize the human gastrointestinal tract and some of the sources included diarrheal patients, no direct link with diarrhea has been established.

The third, taxonomically defined species of *Providencia*, *Providencia heimbachae*, was first described by Muller 1986 (Muller et al., 1986b) who isolated it from penguin feces and from an aborted bovine fetus. A strain of this species recently has been isolated from humans, specifically, the stool of a patient with idiopathic diarrhea (Mohr O'Hara et al., 1999). It should be noted, however, that this strain possesses important biochemical and physiological differences compared to the type strain of Muller (Muller et al., 1986b). While the type strain is positive for phenylalanine deaminase, gas production from glucose and fermentation of maltose and D-mannitol, the human isolate is negative for these tests. Furthermore, motility is only observed after 6 days for the human isolate, compared with 4 days for the type strain.

### Isolation and Identification

Isolation of *Providencia* sp. is routinely performed using bacteriological nutrient media for the general identification of *Enterobacteriaceae*, and, together with the biochemical tests shown in Table 2, to discriminate this species from other enteric forms. Machtiger et al. (1971) found that *P. stuartii* and *P. alcalifaciens* required five amino acids (isoleucine, leucine, valine, glutamic acid and cystine), as well as niacin, for growth on minimal medium containing glucose as a carbon source (Machtiger and O'Leary, 1971). Hawkey et al. (1982b) used a selective and differential enrichment medium to isolate low numbers of *P. stuartii*. Thaller et al. (1992) used a modified MacConkey containing methyl green phosphatase (MGP) to successfully identify 100% of *P. stuartii* isolates from 1,278 seeded

urine samples. By comparison, standard MacConkey medium and the API20E (API System, Bio Merieux Vitec, Inc. Hazelwood, MO.) were only able to identify 82.5% of the same isolates. *Providencia stuartii* and *M. morgani* are the only phosphatase-positive members of the *Enterobacteriaceae*, and MGP distinguishes phosphatase producing colonies on Luria-Bertani-agar by their green pigmentation or halo (Pompei et al., 1990; Satta et al., 1979), while on MacConkey medium the same colonies appear red. Thaller et al. (1992) also added methyl blue to MCP to distinguish lactose-positive colonies (violet) from the phosphatase producers.

A simplified method for the identification of *P. alcalifaciens* has been described by Senior (1997). This method relies on the probability that *P. alcalifaciens* is the only oxidase-negative organism likely to be present in fecal cultures enriched in tetrathionate broth, which also is unable to ferment the mannitol, xylose and galactose present in the medium. When grown on this agar-solidified medium, colonies of *P. alcalifaciens* appear red, in contrast to the colorless colonies of non-*P. alcalifaciens* bacteria that ferment the three sugars. Extensive tests by Senior showed the medium to be both highly specific and sensitive in detecting *P. alcalifaciens* (Senior, 1997). A number of commercial kits have become available for identification purposes and have been utilized, with varying success, for identification of *P. rustigianii* (Kitch et al., 1994; Piccolomini et al., 1991).

### Ecology

The emergence of *P. stuartii* as a significant hospital pathogen since the 1970s has led to efforts to uncover the natural sources and reservoirs of this species. Early work had discounted the gastrointestinal tract as a potential site owing to the lack of isolates obtained using traditional fecal culture methods; however, Hawkey et al. (1982a) demonstrated fecal colonization by *P. stuartii* using a combination of pre-enrichment and selective media. Moreover, the long-term-catheterized human urinary tract appears to offer an attractive niche to this species. Results from studies of patients catheterized for long periods indicate that *P. stuartii* can often be found on catheter surfaces as frequently as more familiar uropathogens such as *E. coli*, *P. mirabilis*, *Enterococcus* sp., and *P. aeruginosa* (Warren, 1986). It also has been isolated from burn and wound infections and bacteremias, which further emphasizes the pathogenicity of this species (Penner, 1984).

*Providencia rettgeri* similarly has emerged in recent years as a nosocomial pathogen of clinical importance. This species has been isolated with

regularity from the urinary tract of catheterized or immunocompromised patients and less frequently from human feces, bile and sputum (Bauernfeind and Wiersma, 1977; Cipriani et al., 1988; Gunalp, 1979; Mino et al., 1997). Of the remaining species, *P. alcalifaciens* is an intestinal colonizer and a recognized cause of gastroenteritis (Janda et al., 1998). *Providencia rustigianii* also has been confirmed as a gastrointestinal tract inhabitant and often is found in the intestinal tract of mammals, such as humans and pigs, and even in arctic birds, e.g., penguins. The true ecological niche inhabited by *P. heimbachae* has yet to be determined, but the evidence at hand suggests that it may inhabit the gastrointestinal tract, as is common for other *Providencia* species (Costas et al., 1987; Higashitani et al., 1995; Mohr O'Hara et al., 1999).

### Epidemiology

Both the indirect hemagglutination test and the indirect hemagglutination inhibition test have been used to type *Providencia* sp (Levina et al., 1980). These tests are frequently useful in distinguishing between individual *Providencia* serogroups (Levina et al., 1980). Antigenic serotyping methods based on the flagellar (H), LPS (O) and capsular (K) antigens also have been applied to *P. stuartii* strains. Such serotyping may be particularly useful in identifying specific strains endemic in different hospitals. Penner and colleagues have used O serotyping successfully (Penner and Hennessy, 1979a; Penner and Hennessy, 1979b; Penner et al., 1979c) to type strains from *P. stuartii*, *P. rettgeri* and *P. alcalifaciens* (Penner and Hennessy, 1979a; Penner and Hennessy, 1979b). In the O serotyping of *P. stuartii* isolates, Penner and his colleagues found that 97% of 829 isolates tested fell into one of 14 O-antigen serotypes. However, the somatic (O) antigen serotyping scheme for 54 isolates of *P. rettgeri* based upon a set of 93 O-antigens, also developed by Penner and Hennessy (1979b), failed to detect a single predominant serotype. While the small size of the strain pool may have influenced this latter result, it is possible that infection by *P. rettgeri* is not due to a few serotypes, as has been found in is much less strain specific than that by *P. stuartii*. Similarly, in the serotyping of *P. alcalifaciens*, Penner (1979c) detected 29 serotypes among 82 typeable isolates. Serotyping schemes for the remaining species of *Providencia* have yet to be developed but will undoubtedly employ similar approaches to those used for other *Providencia* species.

Bacteriophage and bacteriocin typing methods for *Providencia* sp. have yet to be developed, though a number of studies have looked for and isolated bacteriophages from both *P. stuartii* and *P. rettgeri* (Coetzee, 1967; Gabrilovich et al.,

1998; McHale et al., 1981a). A bacteriocin typing scheme has been tested on >300 *Providencia* sp. isolates, though no follow-up confirmation of its reliability has been published (Al-Jumaili and Fenwick, 1978).

Molecular methods of epidemiological typing, such as restriction fragment length polymorphism (RFLP) and ribotyping, have been used with *P. stuartii* and *P. alcalifaciens*. Owen et al. (1988) noted that the DNA restriction fingerprints for *P. stuartii* were quite distinct from species of the allied genera of *Providencia* and *Proteus*, and provided a more sensitive measure of minor genomic differences than total DNA digests. Rahav et al. (1994) used RFLP to demonstrate that a single strain of *P. stuartii* persisted in the same patient during a nursing home outbreak of *P. stuartii* bacteriuria, and that several different strains were responsible for the outbreak. In comparison, neither biochemical tests nor antibiotic sensitivity were able to distinguish separate strains during this outbreak. Guth et al. (1999) used clonal analysis based on ribotyping to show that diarrheal isolates of *P. alcalifaciens* were clustered into two main groups.

The application of genetic methods to the identification of bacteria has become routine in the past decade and this is reflected in the current methods being applied to identification of *Providencia* sp. Apart from classical ribotyping, other methods that potentially could be used to distinguish strains of *Providencia* include automated ribotyping, RAPD-PCR (Akopyanz et al., 1992), amplification and restriction analysis of the 16S rRNA gene (ARDRA; Andrighetto, 1998; Dijkshoorn, 1998) and multilocus sequence typing (MLST), which exploits the electronic portability of nucleotide sequence data (Maiden et al., 1998).

### Pathogenicity

The *Providencia* species that have been clearly identified as pathogens are *P. stuartii*, *P. rettgeri* and *P. alcalifaciens*. In human pathogenicity, the most significant member of the genus is *P. stuartii*, whereas the virulence of *P. rettgeri* and *P. alcalifaciens* is less clear. *Providencia stuartii* is particularly effective in colonizing urinary catheters and is a leading risk factor for bacteremia (Muder et al., 1992; Rahav et al., 1994; Rudman et al., 1988; Woods and Watanakunakorn, 1996). It has been proposed that *P. stuartii*, as well as *P. mirabilis* and *M. morgani*, probably establish a niche within the urinary catheter, thus increasing their ability to cause subsequent bladder bacteriuria (Warren, 1987a). *Providencia stuartii* is not particularly invasive; however, certain circumstances tend to increase the probability of infection, including prolonged catheterization and urinary surgery. This species also is resistant

to many common antibiotics, including most penicillins, aminoglycosides, tetracyclines, older cephalosporins, sulfamethoxazole and fosfomicin (Gomez-Lus et al., 1977; Paradise et al., 1998; Rather et al., 1997; Stock and Wiedemann, 1998; Swiatlo and Kocka, 1987). Such antibiotic resistance gives *P. stuartii* an opportunistic advantage in nosocomial patients. Furthermore, *P. stuartii* also has been implicated in septicemia (bacteremia), with symptoms typical of other septicemias, except that vascular collapse is not a prominent feature (McHale et al., 1981b; Prentice and Robinson, 1979).

While cases of *P. stuartii* septicemia usually prove fatal due to antibiotic resistance, patient survival in response to medical therapy has been reported in individual case studies (Keren and Tyrrel, 1987) and more recently, in long-term epidemiological surveys (Muder et al., 1992; Woods and Watanakunakorn, 1996). In the latter case, Woods and Watanakunakorn (1996) found a mortality rate of 25% in a review of 49 cases of *P. stuartii* bacteremia. Considering that 51% of patients in this survey were infected by more than one bacterial species (polymicrobial bacteremia), the mortality rate for *P. stuartii* bacteremia alone is probably lower. The main differences between *P. stuartii* and *P. rettgeri* are at the biochemical level. *Providencia rettgeri* can metabolize the sugars D-arabitol, adonitol and erythritol (Table 2). Otherwise, most *P. rettgeri* strains exhibit pathogenic properties similar to those of *P. stuartii*. *Providencia rettgeri* UTIs in catheterized and otherwise compromised patients are also difficult to treat owing to multiple antibiotic resistance. Overall resistance is, however, less marked in this species than in *P. stuartii*. By way of example, *P. rettgeri* is particularly susceptible to the aminoglycosides gentamicin and tobramycin, whereas *P. stuartii* is highly resistant to both (Penner and Preston, 1980b; Penner et al., 1982; Piccolomini et al., 1987).

In contrast to *P. stuartii* and *P. rettgeri*, *P. alcalifaciens* is an invasive enteric pathogen and implicated as a cause of diarrheal disease (Albert et al., 1992; Albert et al., 1998; Guth and Perrella, 1996; Haynes and Hawkey, 1989; Sen, 1962). In studies using pure cultures derived from stool specimens that were inoculated into the ilea of adult rabbits with removable ileal ties (RITARD model; Davis, 1991; Spira, 1981), it has been shown that the development of diarrhea is accompanied by an intestinal histopathology typical of other invasive bacterial species, such as *Shigella flexneri* (Albert et al., 1992). Mathan et al. (1993) have added more evidence to the *P. alcalifaciens* virulence model, invasiveness model by demonstrating two modes of bacterial entry into epithelial cells. The first mode of entry is by direct endocytosis associated with polymerization of cytoskeletal components, and the sec-

ond mode by which the bacteria enter is through disruption of tight junctions, with the bacteria entering into and proliferating in intercellular spaces. The invasive abilities of *P. alcalifaciens* have been tested in HEp-2 cells (an epithelioid cell line from a human laryngeal carcinoma) by two independent scientific groups (Albert et al., 1992; Janda et al., 1998). Both groups were able to confirm penetration by *P. alcalifaciens* isolates, whereas no strain of *P. stuartii* or *P. rettgeri* tested invaded the HEp-2 cells.

**VIRULENCE FACTORS.** Those contributing to the pathogenicity of *Providencia* sp. that have been investigated include cellular adherence, the production of fimbriae and of urease. *Providencia stuartii* has been the focus of the majority of research.

**Adherence and fimbriae.** Urinary tract infections (UTIs) due to *P. stuartii* persist longer than those due to other Gram-negative bacteria. It has been suggested that a possible reason for this increased persistence may be due to the ability of *P. stuartii* to adhere to uroepithelial cells (Mobley et al., 1986). Adherence to uroepithelial cells can be enhanced by the expression of MR/K (hemagglutinin)-type fimbriae (see "Fimbriae"). Mobley et al. (1988) found that a significant proportion of *P. stuartii* isolated from patients experiencing bacteriuria of long duration expressed MR/K fimbriae. These data implicate the MR/K hemagglutinin in an important role in UTI persistence of *P. stuartii*.

**Urease.** While *Providencia rettgeri* is urease positive, as are some strains of *P. stuartii*. In fact, *P. rettgeri* and *P. stuartii* were originally subdivided based on urease production (Penner et al., 1976). There is some evidence that urease-producing strains of *P. stuartii* are more likely to be involved in long-term colonization of urinary catheters with subsequent development of blockages due to stone formation (Kunin, 1989; Stickler et al., 1998). Urease-producing *P. stuartii* strains also have been found to inhibit the in vitro growth of *E. coli* on catheter surfaces and in urine (Fletcher et al., 1994). This adaptive advantage in growth may explain why *P. stuartii* are the dominant species isolated in these mixed infections. The construction of isogenic urease-negative mutants may help to better define the role of urease in *P. stuartii* pathogenesis.

## Conclusion

The genera *Proteus*, *Morganella* and *Providencia* contain a number of important human pathogens that often cause serious infections in hospitalized and immunocompromised patients. Other species are normal intestinal flora in animals or animal pathogens. One of the most conspicuous features of members of the genus *Proteus* is their

ability to differentiate into elongated, multinucleated swarmer cells upon contact with a solid surface. Methods of isolation and identification have been developed for most species and these now include several DNA-based methods of detection; however, treatment of infected patients is often problematic owing to the development of antibiotic resistance and the immunocompromised state of the patient.

The information contained in this chapter gives the reader a broad understanding of the current state of knowledge concerning these related genera. There are a number of areas where this knowledge base needs strengthening in the future. For example, our understanding of the ecological and/or pathogenic role of several species, including *P. myxofaciens*, *P. rustigianii* and *P. heimbachae*, has advanced very little since their initial identification, and much work remains to be done in this regard.

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## *Y. enterocolitica* and *Y. pseudotuberculosis*

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### **Enteropathogenic *Yersinia*: Introduction (Elisabeth Carniel)**

The genus *Yersinia* belongs to the family Enterobacteriaceae and is currently composed of 11 species. Three of them are pathogenic for humans and animals: the enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica* and the plague agent *Y. pestis*. This chapter deals with the two enteropathogenic species, while *Y. pestis* is described in the *Yersinia Pestis* and Bubonic Plague in this Volume.

#### Taxonomy

Van Loghem in 1944 (Van Loghem, 1944) proposed the separation of the genus *Yersinia* from the genus *Pasteurella*, which became effective in 1974 (Mollaret and Thal, 1974).

*Yersinia pseudotuberculosis* was first described by Malassez and Vignal in 1883 (Malassez and Vignal, 1883; Malassez and Vignal, 1884). This bacterium received numerous names (Mollaret, 1965): bacille de la tuberculose zoogléique, *Bacillus pseudotuberculosis*, *Bacterium pseudotuberculosis rodentium*, *Pasteurella pseudotuberculosis*, before being finally designated *Y. pseudotuberculosis* in 1974 (Mollaret and Thal, 1974).

*Yersinia enterocolitica* was first described in 1939 by Schleifstein and Coleman (Schleifstein et al., 1939) who called it *Bacterium enterocoliticum* in 1943 (Schleifstein and Coleman, 1943). Different names were subsequently given to this organism: *Pasteurella pseudotuberculosis*-like, *Pasteurella pseudotuberculosis* type B, *Pasteurella* X, *Pasteurella* Y, Germe X, and finally *Y. enterocolitica* in 1964 (Frederiksen, 1964). It appeared later on that this species was heterogeneous and contained several related species designated “*Y. enterocolitica*-like” (Brenner et al., 1980b). Taxonomical studies allowed the separation of *Y. enterocolitica* sensu stricto (Bercovier et al., 1980a) from *Y. enterocolitica*-like species: *Y. intermedia* (Brenner et al., 1980a), *Y. kristensenii* (Bercovier et al., 1980b), *Y. fredericksonii*

(Ursing et al., 1980), *Y. aldovae* (Bercovier et al., 1984) and *Y. rohdei* (Aleksic et al., 1987). These widespread environmental species are usually not associated with disease. More recently, *Y. mollaretii* and *Y. bercovieri* were also separated from *Y. enterocolitica* (Wauters et al., 1988b). These two species formerly belonged to biogroups 3A and 3B of *Y. enterocolitica*, respectively. Their importance in pathogenesis is for the moment unknown, but they seem to be devoid of virulence-linked properties. *Yersinia ruckeri* (Ewing et al., 1978; De Grandis et al., 1988b) is an important fish pathogen responsible for the red mouth disease in rainbow trout and some other fish. However, its classification in the genus *Yersinia* is controversial.

Like other members of the family Enterobacteriaceae, *Y. enterocolitica* and *Y. pseudotuberculosis* are Gram-negative, aero-anaerobic rods. They still have some particularities that distinguish them from the other members of this family. They are motile at temperatures inferior to 30°C only, they form colonies in 48 h instead of 24 h, their optimal growth temperature is around 30°C, they are able to multiply at low temperatures (4°C), and they have a G+C content (46–49 mol%) lower than that of most members of this family.

#### History

DNA/DNA hybridization studies performed in the 1980s demonstrated that *Y. enterocolitica* is the most distantly related pathogenic species of *Yersinia* (Bercovier et al., 1980a). *Yersinia pestis* and *Y. pseudotuberculosis* exhibit such a high degree of DNA relatedness that they should have been included into a single species (Bercovier et al., 1980b). However, *Y. pestis* causes plague and is transmitted by fleabites, whereas *Y. pseudotuberculosis* is transmitted by the fecal-oral route and rarely leads to death. Because of the historical importance of *Y. pestis* in human history and for the sake of security, the reclassification of *Y. pestis* and *Y. pseudotuberculosis* into a single species has been rejected.



A more recent analysis of the three pathogenic species by multilocus sequence typing (MLST) indicated that the separation between *Y. pestis*/*Y. pseudotuberculosis* and *Y. enterocolitica* occurred 42–187 million years ago, and that the last common ancestor of *Y. pestis*/*Y. pseudotuberculosis* existed 0.4–1.9 million years ago (Achtman et al., 1999). *Yersinia pestis* was found to be a clone that evolved from *Y. pseudotuberculosis* 1500–20,000 years ago, shortly before the first known pandemics of human plague. Despite their long existence, the enteropathogenic species *Y. enterocolitica* and *Y. pseudotuberculosis* were not known before the end of the nineteenth century.

*Yersinia enterocolitica*, first described in the United States in 1939, was subsequently isolated in Europe some ten years later. Before 1966, the organism was isolated from a wide variety of animal species, but no more than 23 cases of human infection were reported in the literature worldwide. However, this number dramatically increased over the next years and *Y. enterocolitica* is now found all over the world, with a higher incidence in temperate and cold countries. The first major foodborne human outbreak of this organism was reported in the United States in 1976, but *Y. enterocolitica* is most often responsible for sporadic cases.

*Yersinia pseudotuberculosis* was identified in 1883, before *Y. enterocolitica*. This species infects a wider range of animals than *Y. enterocolitica* but its importance in human infections is much lower than that of *Y. enterocolitica* worldwide.

Both species are transmitted by the oral route and cause intestinal symptoms such as abdominal pain (especially *Y. pseudotuberculosis*), diarrhea (especially *Y. enterocolitica*), and fever.

The epidemiology, clinical features, immunology, virulence factors, physiopathology, bacterial diagnosis, and treatment of these two species will be described in the various paragraphs of this chapter.

## ***Yersinia enterocolitica***

### **Epidemiology (Elisabeth Carniel)**

That *Yersinia enterocolitica* is a global pathogen has been suggested by numerous reports, which are summarized in different reviews (Mollaret, 1976; Swaminathan et al., 1982; Cover and Aber, 1989; Bottone, 1997; Bottone, 1999; Naktin and Beavis, 1999).

**GEOGRAPHICAL DISTRIBUTION** *Yersinia enterocolitica* has been isolated in various countries on all five continents.

Several countries in Asia: Bangladesh (Butler et al., 1984; Carniel et al., 1986), China (Seto and

Lau, 1984; Ding et al., 1986; Zheng and Xie, 1996; Fukushima et al., 2001a), India (Abraham et al., 1997), Iran (Haghighi, 1979), Israel (Shmilovitz and Kretzer, 1978), Japan (Kanazawa and Ikemura, 1979; Kato et al., 1985; Saitoh et al., 1994; Hosaka et al., 1997), and Turkey (Gonul and Karapinar, 1991).

All European countries including Belgium (Wauters, 1972; Wauters, 1979; Mollaret et al., 1979; Vandepitte and Wauters, 1979; Wauters and Vandepitte, 1982; Verhaegen et al., 1998), Finland (Hamama et al., 1992), Czechoslovakia (Rakovsky et al., 1973), France (Alonso et al., 1979; Mollaret et al., 1979; Servan et al., 1979; Mollaret and Alonso, 1988), Germany (Nesbakken, 1988), Hungary (Szita and Svidro, 1976), Norway (Kapperud, 1981; Saebø and Lassen, 1992; Kapperud and Vardund, 1995), Russia (Iushchenko et al., 1982; Sharapova and Alekseeva, 1983; Gurleva et al., 1985; Pavlov, 1985), and Sweden (Hurvell et al., 1979).

South and North America: Argentina (De Centorbi et al., 1989; Escudero et al., 1991), Brazil (Pizzolitto et al., 1979), Canada (Lafleur et al., 1972; Lafleur et al., 1979; Toma, 1973; Toma and Lafleur, 1974; Toma et al., 1979; Schiemann and Fleming, 1981b; Hariharan et al., 1995; Letellier et al., 1999), and the United States (Eden et al., 1977; Bottone and Robin, 1979; Shayegani et al., 1979; Marymont et al., 1982; Walker and Grimes, 1985; Naqvi et al., 1993).

A few places in Africa: Morocco (Hamama et al., 1992), South Africa (Jennings et al., 1987), and West Africa (Anjorin et al., 1979).

And in Oceania: New Zealand, Australia (Saitoh et al., 1994).

The incidence of yersiniosis is higher in temperate and cold countries. *Yersinia enterocolitica* was found to be the causative agent of 2.8% of the enteritis in Montreal (Marks et al., 1980), 2.9% in the Netherlands (Hoogkamp-Korstanje et al., 1986), and 1.4% in Italy (Mingrone et al., 1987). In Norway, this species was the fourth leading cause of acute enteritis (1%) after *Salmonella* (5%), *Campylobacter* (3%) and *Shigella* (3%). In Belgium, *Y. enterocolitica* was found to be the third most common bacterial enteropathogen after *Salmonella* and *Campylobacter* (Verhaegen et al., 1991). In New York State, *Y. enterocolitica* was isolated from 10 to 25% of 275 water, milk, and animal specimens examined and from about 2.3% of 300 sequential stool specimens submitted for *Salmonella* and *Shigella* screening (Shayegani et al., 1979).

Bioserotype 4/O:3 strains are the most common pathogenic *Yersinia enterocolitica* found over the world (Mollaret et al., 1979; Marriott, 1987; Mingrone et al., 1987), followed by 2/O:9 strains. The phage type of 4/03 strains differentiates them into three main subgroups: phage type

VIII, which is widespread in Europe and in many other countries; phage type IXa, which is found essentially in South Africa and Hungary; and phage type IXb, which is restricted to Australia, New Zealand, the United States, and Canada (Nicolle et al., 1976; Pham et al., 1995; Bottone, 1997).

Bioserotype 2/O:9 strains are the major cause of *Y. enterocolitica* diarrhea in the United Kingdom (Prentice et al., 1991).

*Yersinia enterocolitica* 1B/O:8 were restricted to North America and were the predominant bioserotype in the United States in the past few decades (Kay et al., 1983); however, these strains are now isolated in other parts of the world and their importance decreased in the United States in the mid- to late 1980s (Bottone et al., 1987; Bissett et al., 1990). The major bioserotype found in this country is now 4/O:3.

**RESERVOIRS** A wide variety of ecological niches may shelter *Y. enterocolitica*.

The environment (soil and water; Wauters, 1972; Kapperud, 1977; Botzler, 1979; Botzler, 1987; Iushchenko, 1982; Walker, 1985; Ziegert, 1990; Gonul, 1991; Saitoh, 1994)

Food (milk, retail meat products, vegetables, eggs, cheese, etc.; Iushchenko et al., 1982; Pavlov, 1985; Ding et al., 1986; Schiemann, 1987; De Centorbi et al., 1989; Hamama et al., 1992; Durisin et al., 1997)

A wide range of animals: domestic (cat and dog), stock farm (chinchilla, mink, pig, rabbit, cow, goose, horse, sheep, and buffalo), zoo (monkey), wild (raccoon, fox, snail, frog, beaver, deer, ocelot, crab, flies, and fleas), birds (robin), shellfish (oyster), and many species of small rodents (McClure et al., 1971; Wauters et al., 1971; Alonso and Bercovier, 1975; Toma and Deidrick, 1975; Wauters and Janssens, 1976; Kapperud, 1977; Servan et al., 1979; Iushchenko et al., 1982; Gurleva et al., 1985; Kato et al., 1985; Walker and Grimes, 1985; Shayegani et al., 1986; Weber et al., 1987; Cox et al., 1990; Escudero et al., 1991; Weynants et al., 1996; Zheng and Xie, 1996; Funk et al., 1998; Letellier et al., 1999; Naktin and Beavis, 1999)

Most of the strains isolated from the environment belong to the nonpathogenic biotype 1A, with the exception of pathogenic strains of biotype 1B, which are frequently isolated from water.

Pathogenic *Y. enterocolitica* of biotypes 2 to 5 are mainly isolated from animals (sheep, cattle, goats, and poultry) or food. There is a preferential relationship between the bioserotype of the strains and their ecological niches (Mollaret, 1976; Bottone, 1997): 4/O:3 strains are isolated with a high frequency from pigs; 2/O:9 strains are often found in cows and goats; the reservoir of

strains of bioserotypes 2 or 3/O:5,27 (the third most common *Y. enterocolitica* pathogen in Europe after 4/O:3 and 2/O:9 strains) is not clearly established; strains of the very rare bioserotypes 5/O:2,3 are isolated from sheep, hares and goats (Slee and Button, 1990), and 3/O:1,2a,3 from chinchillas (Bercovier et al., 1980a).

**TRANSMISSION** Transmission of *Y. enterocolitica* occurs by the fecal-oral route. The organisms are ingested with contaminated food. After their journey in the intestinal tract of their host (where they exert their pathogenic effect; Physiopathology of *Y. enterocolitica* and to Clinical symptoms of *Y. enterocolitica*), they are excreted in the feces, which can serve as a source of contamination of a new host or of the environment.

Children below the age of five are much more prone to *Y. enterocolitica* infections (Mollaret, 1976; Cover and Aber, 1989) because they have weaker defense mechanisms, immature bacterial flora in their intestines, behaviors favoring bacterial infections (crawling and suckling), and surveillance of diarrhea is more intensive in infants than in adults.

Peaks of human yersinioses have been reported to predominate during the cold season (Mollaret, 1976; Swaminathan et al., 1982); however, this does not appear to be the case over the last years in France where the majority of the strains (including pathogenic ones) are predominantly isolated during the summer (French National Reference Laboratory, unpublished data).

*Sporadic Human Cases Yersinia enterocolitica* 4/O:3 and 2/O:9 most often cause isolated cases or small family outbreaks, although they have been implicated in a few community outbreaks.

One of the major sources of human infection with pathogenic *Y. enterocolitica* 4/O:3 is through the handling of swine and the consumption of pork meat (Tauxe et al., 1987). Pigs are known to carry the organism in their oropharynx, nasopharynx, and their intestine. Contamination by *Y. enterocolitica* of pigs and of pig carcasses in slaughterhouses has been largely documented (Toma and Deidrick, 1975; Wauters and Janssens, 1976; Nicol, 1978; Asakawa et al., 1979; Hurvell et al., 1979; Doyle et al., 1981; Schiemann and Fleming, 1981b; Harmon et al., 1984; Walker and Grimes, 1985; Andersen, 1988; Nesbakken, 1988; De Boer et al., 1991; Trallero et al., 1992; Hariharan et al., 1995; Letellier et al., 1999; Naktin and Beavis, 1999). For instance, in one study (Andersen, 1988), 24.7% of 360 freshly slaughtered pig carcasses were found infected with *Y. enterocolitica* 4/O:3. The source of contamination was the intestinal content of the pigs.

The use of a mechanized bung cutter in connection with enclosing the anus and rectum in a plastic bag to minimize fecal contamination was recommended to reduce infection of pork meat. In Norway, up to 83% of slaughtered pigs were carriers of high numbers of *Y. enterocolitica* 4/O:3 on their tongue and tonsils (Nesbakken, 1988). Consumption of raw or undercooked infected meat may then cause human infections (Tauxe et al., 1987; Stoddard et al., 1994; Kellogg et al., 1995). The infected meat may also serve as a source of contamination of other food products by direct contact with these products or via infected knives, hands, or recipients.

Cows, goats and sheep may also be a source of human infection with *Y. enterocolitica*, but the bioserotype of these isolates is most often 2/O:9. This represents a major problem for stock farmers because antibodies against serotype O:9 crossreact with *Brucella* antigen (Weynants et al., 1996; Gerbier et al., 1997; Garin-Bastuji et al., 1999), and suspicion of brucellosis should lead to the elimination of the animals according to public health regulations. It is not yet clear whether human infections with *Y. enterocolitica* 2/O:9 occur directly by handling the animals or by eating their contaminated meat, or indirectly by the consumption of vegetables infected by the animal feces.

Infected greenery may thus be a primary source of human infection. The ability of *Yersinia* strains to multiply at 4°C is an important factor to take into consideration. Storage of food in refrigerators was probably one of the major reasons for the sudden "explosion" of yersiniosis cases in the 1950s. Foodstuffs infected with a low number of pathogenic *Y. enterocolitica* may not cause any symptom if consumed immediately, while the same product kept in the refrigerator will be heavily loaded with *Yersinia* after a few days, and may then cause a disease. The inoculum of *Y. enterocolitica* required to cause intestinal symptoms has been determined to be 10<sup>9</sup> bacteria/ml (Edelman and Levine, 1980).

Person-to-person transmission, although scarcely documented, probably represents another mode of circulation of pathogenic *Y. enterocolitica* through infected hands. Family outbreaks may be due to consumption of a common infected meal but the sequential onset of illness in family members also suggests a possible person-to-person transmission (Ahvonen, 1972; Gutman et al., 1973; Fukushima et al., 1981; Martin et al., 1982). Excretion of *Y. enterocolitica* in the stools of children with enteritis may last 14–97 days (Marks et al., 1980). A few cases of nosocomial infections have been reported (Toivanen et al., 1973; Ratnam et al., 1982; McIntyre and Nnochiri, 1986). However, the isolation of nonpathogenic strains of biotype 1A in two of these three out-

breaks suggests that these isolates were probably not the cause of the symptoms.

Strains of bioserotype 1B/O:8 may also be, although seldom are, responsible for sporadic cases of human infections following ingestion of contaminated water (Keet, 1974).

**LARGE COMMUNITY OUTBREAKS** Large human outbreaks due to *Y. enterocolitica* are most often associated with strains of bioserotype 1B/O:8 and have been reported only in the United States (Bottone, 1997). The first major human foodborne outbreak of *Y. enterocolitica* O:8 infection occurred in New York State in 1976, among children and employees of several schools who had consumed chocolate milk contaminated with this bacterium (Black et al., 1978). Another outbreak due to absorption of powdered milk was reported in 1981 in a summer camp in New York (Shayegani et al., 1983). The same year, the consumption of tofu washed with contaminated water resulted in an outbreak caused by *Y. enterocolitica* O:8 in Washington (Tacket et al., 1985). Contaminated water used to wash bean sprouts was also responsible for a *Y. enterocolitica* O:8 outbreak in a Brownie troop in Pennsylvania in 1982. Recently another outbreak due to *Y. enterocolitica* 1B/O:8 occurred in Vermont and New Hampshire and was associated with consumption of bottled pasteurized milk from a local dairy (Ackers et al., 2000). This outbreak likely resulted from post-pasteurization contamination of milk.

However, other bioserotypes of *Y. enterocolitica* have been associated in some rare instances with human outbreaks. This was the case among people who participated in a feast in Tamil Nadu village (India) where buttermilk contaminated with a 4/O:3 strain was consumed (Abraham et al., 1997). An outbreak associated with the household preparation of chitterlings contaminated with a strain of the same bioserotype was also reported in Georgia in 1989 (Lee et al., 1990). Finally, contamination of pasteurized milk with a *Y. enterocolitica* isolate of serotype O:13a,13b caused a large outbreak in three states in the United States in 1982 (Tacket et al., 1985).

A few other foodborne outbreaks have been suspected to be due to *Y. enterocolitica*, although the role of this species as the causal agent could not be confirmed. Infected water has been reported to be the source of a *Y. enterocolitica* outbreak involving 41% of the visitors and employees who were at a ski resort in Montana (Eden et al., 1977).

**MOLECULAR EPIDEMIOLOGY** *Yersinia enterocolitica* strain differentiation has relied, until recently, on phenotypic characteristics (biotype, serotype, and phage type). Since these markers exhibit a

very low degree of polymorphism, the establishment of an epidemiological link between two strains based on their sharing the same bioserotype is not fully satisfactory. Recent advances in enzymatic (multi-locus enzyme electrophoresis [MLEE]) and genetic (restriction endonuclease analysis of the plasmid [REAP], ribotyping, restriction enzyme digestion of the chromosome [REAC], polymerase chain reaction [PCR]-fingerprinting, and pulsed-field gel electrophoresis [PFGE]) techniques have provided a means to reevaluate the polymorphism of isolates within a given bioserotype, and therefore to trace the spread of some clones over the world or during a local outbreak.

*Multi-locus Enzyme Electrophoresis* Analyses of the electrophoretic metabolic enzyme polymorphism of *Yersinia* spp. have shown that *Y. enterocolitica* strains belong to one cluster and that subgroups within this cluster most often match the bioserotypes of the isolates (Gouillet and Picard, 1984; Gouillet and Picard, 1988; Dolina and Peduzzi, 1993). However, within one bioserotype of *Y. enterocolitica*, the discriminatory power of MLEE was not satisfactory because strains of the same serotype were also of the same electrophoretic type (ET) and strains of different serotypes could be of the same ET and thus could not be distinguished.

*Restriction Endonuclease Analysis of the Plasmid* In this technique, the pYV virulence plasmids present in all pathogenic *Yersinia* (pYV) are extracted, digested with a restriction enzyme, and their banding patterns are compared. This method has the advantage of being rapid and easy to perform, but it is not applicable to nonvirulent *Yersinia* or to virulent strains spontaneously cured of their pYV (easily observed upon subculture or growth at 37°C).

Several studies have reported the use of REAP to compare *Y. enterocolitica* strains (Heesemann et al., 1983; Wachsmuth et al., 1984; Kaneko and Maruyama, 1987a; Kaneko and Maruyama, 1987b; Kapperud and Nesbakken, 1987a; Nesbakken et al., 1987; Kapperud et al., 1990; Kapperud et al., 1991; Fukushima et al., 1993; Fukushima et al., 1998a; Itean et al., 1996). All studies used the restriction enzymes *EcoRI* and *BamHI* to digest plasmid DNA (and *HindIII* in addition in two studies; Kaneko and Maruyama, 1987a; Kaneko and Maruyama, 1987b). In almost all cases, the plasmid restriction profiles were bioserotype specific, i.e., the REAP pattern was a marker of the bioserotype of the strains. The only exceptions were bioserotypes 2/O:5 and 3/O:3 strains on the one hand (Itean et al., 1996) and bioserotypes 4/O:3 and 3/O:3 strains on the other hand (Fukushima et al.,

1993), which were found to have identical REAP patterns.

Bioserotype 1B/O:8 strains displayed the highest degree of REAP polymorphism since up to six plasmid restriction profiles could be identified in this group (Kapperud and Nesbakken, 1987a; Nesbakken et al., 1987; Kapperud et al., 1990). Strains of serotype O:5,27 (biotypes 2 or 3) exhibited a moderate degree of polymorphism and could be divided into one to three REAP groups (Kapperud and Nesbakken, 1987a; Nesbakken et al., 1987; Kapperud et al., 1990; Fukushima et al., 1993; Itean et al., 1996). Strains of bioserotypes 4/O:3 and 2/O:9 displayed the lower degree of REAP diversity. A single plasmid profile was usually observed among 4/O:3 strains (Heesemann et al., 1983; Kaneko and Maruyama, 1987a; Kaneko and Maruyama, 1987b; Kapperud and Nesbakken, 1987a; Nesbakken et al., 1987; Fukushima et al., 1993; Itean et al., 1996), although two additional plasmid profiles could be identified (in a total of three strains) in one instance (Kapperud et al., 1990). All 2/O:9 strains of worldwide origin studied were highly monomorphic (Heesemann et al., 1983; Kapperud and Nesbakken, 1987a; Nesbakken et al., 1987; Kapperud et al., 1990). Only in two studies could two profiles be distinguished in these strains (Itean et al., 1996; Fukushima et al., 1998a).

It appears that the plasmid profiles reflect quite well *Y. enterocolitica* bioserotypes but that the degree of polymorphism obtained within a given bioserotype is low. The use of REAP for epidemiological tracing of bacteria during an outbreak might thus not bring valuable information.

*PCR-fingerprinting Methods* Randomly amplified polymorphic DNA (RAPD) is based on the random amplification of genomic DNA at low annealing temperatures with arbitrarily selected primers. Application of this technique to various *Y. enterocolitica* isolates showed a relatively weak correlation between their RAPD clusters and bioserotypes, and the technique was not sensitive enough to efficiently separate strains within each bioserotype (Rasmussen et al., 1994; Odinet et al., 1995; Leal et al., 1999).

Degenerate oligonucleotide-primed PCR (DOP-PCR), a more recent PCR method that involves multiple locus priming with evenly dispersed sites within a given genome, was also applied to *Y. enterocolitica* isolates (Sayada et al., 1994). However, the number of strains (eight) studied was too low to draw any conclusion about the usefulness of this technique for molecular epidemiology.

Inter-repeat-PCR (IR-PCR) amplifies specific genomic regions known to be variable among

different prokaryotic genomes. The discriminatory power of this technique, involving amplification of enterobacterial repetitive intergenic sequences, was very poor when applied to *Y. enterocolitica* (Odinot et al., 1995).

**Ribotyping** The ribotyping method is based on the analysis of the restriction fragment length polymorphism generated after hybridization of digested bacterial genomic DNA with an RNA or a DNA 16 + 23 S rRNA probe. This technique was applied to *Y. enterocolitica* by several laboratories, but inter-study comparisons are difficult because of the use of different restriction enzymes: *NciI* and *AvaI* (Andersen and Saunders, 1990), *EcoRI* and *HindIII* (Picard-Pasquier et al., 1990), *NciI* alone (Blumberg et al., 1991), *HindIII*, *NciI*, *BglI*, *SalI*, and *EcoRI* (Mendoza et al., 1996), *EcoRI* and *EcoRV* (Iteman et al., 1996; Fukushima et al., 1998a), and *HindIII* and *BglI* (Lobato et al., 1998). Nonetheless, all studies demonstrated that ribotyping allows to some extent the distinction of subgroups within a given bioserotype, and that the different ribotypes observed are usually bioserotype-specific (i.e., there is a good correlation between phenotypic and ribotypic traits). However, ribotypic polymorphism appears to be higher for strains found in the environment (nonpathogenic strains of biotype 1A or pathogenic strains of biotype 1B) than for those adapted to animal hosts (4/O:3 and 2/O:9).

In a few instances, ribotyping was used to revisit the epidemiological link between strains isolated from a reservoir or a source of infection during an outbreak and those isolated from patients. In one study, no difference in the ribotypes of strains from pigs and human patients could be detected (Andersen and Saunders, 1990), consistent with the hypothesis that pigs are a major reservoir of human yersiniosis. Similarly, the finding of identical ribotype patterns in chitterlings and human specimens from the Atlanta outbreak (Lee et al., 1990) lent further support to the epidemiological evidence that swine were the source of infection and a major reservoir for *Y. enterocolitica* O:3 (Blumberg et al., 1991). However, the fact that only two ribotypes were predominantly found among O:3 United States isolates, and that the ribotypes strictly matched the phage types, indicated that ribotyping did not provide new information compared to classical phenotyping methods in that case. Nonetheless, combination of the patterns obtained with five different restriction enzymes allowed the differentiation of O:3 *Y. enterocolitica* strains from Asturias (Spain) into 11 ribotypes (Mendoza et al., 1996), and the use of only two restriction enzymes (*HindIII* and *BglI*) was sufficient to distinguish nine subgroups. The

two major groups that were found to be endemic in Spain were isolated both from human patients and from commercial raw meat products.

Ribotyping may add some degree of polymorphism to the classical phenotypic markers. However, the limited heterogeneity of the patterns within the most frequent pathogenic bioserotypes suggests that this technique could be useful to trace the spread of *Y. enterocolitica* clones over the world but that it is of more limited interest for outbreak investigations.

**PULSED-FIELD GEL ELECTROPHORESIS** Attempts were originally made to compare the fragment patterns from the total restriction enzyme digestion of chromosomal DNA (REAC) using classical restriction enzymes and electrophoresis systems (Kapperud et al., 1990; Blumberg et al., 1991). However, the complexity of the patterns made interpretation and comparison of the results extremely difficult.

The use of rare cutting restriction enzymes, which yield a moderate number of DNA fragments, in combination with pulsed-field gel electrophoresis, which separates large-size DNA fragments, allows for analyzing and comparing restriction patterns of whole bacterial genomes.

Application of this technique to *Y. enterocolitica* demonstrated that it is by far the most discriminatory method (Buchrieser et al., 1994; Najdenski et al., 1994; Saken, 1994; Iteman, 1996; Asplund, 1998; Fredriksson-Ahomaa, 1999). The restriction enzymes most frequently used for *Y. enterocolitica* were *NotI* and/or *XbaI*. Digestion with *ApaI* and *XhoI* proved also useful in further subdividing strains within a given *NotI* pulsotype (Fredriksson-Ahomaa et al., 1999). All studies demonstrated that strains are genomically related within each bioserotype, and therefore major serotype-specific pulsotypes can be delineated. Furthermore, within each major pulsotype, *Y. enterocolitica* could be separated into numerous subtypes. In countries such as Finland or Denmark where bioserotype 4/O:3 represents up to 90% of all isolated *Y. enterocolitica*, the availability of a technique that allows the differentiation of this group into various subtypes might prove useful during epidemiological investigations; however, most studies show that one or two pulsotypes usually prevail within a geographical area.

Until now, PFGE has been seldom applied to study links between strains in an epidemiological context. The comparison of the pulsotypes of five pairs of strains isolated either from the same patient at different time intervals, or from siblings, demonstrated that in four cases, the two strains of the same pair displayed identical pulsotypes (Najdenski et al., 1994). In the fifth pair, a single restriction fragment differed between

the two strains, suggesting that genomic polymorphism may be generated rapidly under natural conditions in *Y. enterocolitica*.

Comparison of PFGE with other molecular typing methods such as REAP and ribotyping (Iteman et al., 1996) or MLEE (Saken et al., 1994) on the same *Y. enterocolitica* isolates indicated that in all cases, PFGE patterns were the most polymorphic.

### Clinical Manifestations of *Y. enterocolitica* Infections (Mikael Prentice)

**GASTROINTESTINAL ILLNESS** Most infections are in children under 5 years of age (Prentice et al., 1991; Abdel-Haq et al., 2000) presenting with diarrhea, low-grade fever, abdominal pain and vomiting (Marks et al., 1980; Lee et al., 1990). The diarrhea is persistent (14 days; Marks et al., 1980) and in 25–50% of cases contains frank blood (Tacket et al., 1985; Abdel-Haq et al., 2000). Prospective studies show horizontal spread within families (Marks et al., 1980) and horizontal spread can result in nosocomial disease (Cannon and Linnemann, 1992; Epidemiology of *Y. enterocolitica*). Pharyngitis is often reported, particularly in adults (Cover and Aber, 1989), and *Y. enterocolitica* can be grown from throat swabs in some infected patients (Tacket et al., 1983; Rose et al., 1987). Central abdominal and right iliac fossa pain that may simulate appendicitis (pseudoappendicitis) is more common in older children and young adults (Black et al., 1978; Attwood et al., 1987; Cover and Aber, 1989; Prentice et al., 1991). Although these symptoms have been associated with *Y. enterocolitica* infection for over 30 years (Winblad et al., 1966), it is still very difficult (Shorter et al., 1998) for surgeons to distinguish sporadic appendicitis requiring surgery from pseudoappendicitis due to *Y. enterocolitica* infection (for which surgery is not indicated because it usually resolves spontaneously or with antibiotic therapy; Cover and Aber, 1989). At laparotomy, the usual findings are terminal ileitis with enlarged mesenteric nodes with a slightly edematous or inflamed appendix (Winblad et al., 1966; Cover and Aber, 1989; Shorter et al., 1998). *Yersinia enterocolitica* can be grown from the mesenteric nodes and the appendix (Winblad et al., 1966; Verhaegen et al., 1998).

Histology in the severe cases coming to post-mortem shows ulceration and necrosis of the bowel overlying lymphoid follicles extending over the length of the gastrointestinal tract from small intestine (in some cases stomach) to colon (Bradford et al., 1974; Physiopathology). Mesenteric lymph nodes are enlarged showing focal areas of necrosis (Bradford et al., 1974) and infiltration by leucocytes (Winblad et al., 1966;

Bradford et al., 1974). Intestinal perforation and gastrointestinal hemorrhage may rarely occur as a consequence of ulceration (Bradford et al., 1974; Moeller and Burger, 1985). There is some evidence for involvement of *Y. enterocolitica* and *Y. pseudotuberculosis* in granulomatous appendicitis, a histopathological diagnosis made in 0.1–2% of appendectomies (Lamps et al., 2001). The PCR for *Yersinia*-specific targets (either *Y. pseudotuberculosis* or *Y. enterocolitica*) was positive in 25% of a series of appendectomy specimens containing granulomas (Lamps et al., 2001).

Primary *Y. enterocolitica* peritonitis secondary to liver disease with infected ascites (Reed et al., 1997) has also been reported. *Yersinia enterocolitica* has been associated with intussusception (Burchfield et al., 1983; Winesett et al., 1996). Chronic and relapsing gastrointestinal disease with symptoms lasting for several years has been reported (Hoogkamp-Korstanje et al., 1988) where *Y. enterocolitica* could not be cultured but was persistently demonstrated by immunofluorescence in the intestinal mucosa and associated lymph nodes.

**SYSTEMIC DISEASE Septicemia** Iron-overloaded patients (e.g., those with thalassemia [Chiu et al., 1986; Adamkiewicz et al., 1998], sickle cell disease [Blei and Puder, 1993], or hemochromatosis [Prentice et al., 1991]), patients on chronic hemodialysis (Boelaert et al., 1987), those following acute iron supplement overdose (Bouza et al., 1980; Melby et al., 1982; Prentice et al., 1991), those with liver disease (Bouza et al., 1980) or diabetes (Bouza et al., 1980), and the elderly are at increased risk of a septicemic illness with a fatal outcome (Bullen et al., 1991; Prentice et al., 1991; Other putative or confirmed virulence factors, iron acquisition systems). It has been estimated that patients with, for example,  $\beta$ -thalassemia, are 5000 times more likely to have nonenteric *Y. enterocolitica* infection than the general population (Adamkiewicz et al., 1998). Medical interventions associated with systemic *Y. enterocolitica* infection include the use of the iron-chelating agent desferrioxamine (Robins-Browne and Prpic, 1983; Hoen et al., 1988), which provides an additional risk beyond that of the underlying iron-overload (Boyce et al., 1985) by virtue of *Y. enterocolitica* growth stimulation (Robins-Browne and Prpic, 1985a) and host immunosuppression (Autenrieth et al., 1994b). Conjugation of desferrioxamine with starch may provide a safer chelating agent (Schubert and Autenrieth, 2000a).

A low-grade bacteremia may result in seeding of particular intravascular foci producing a continuous bacteremia. Mycotic aneurysm due to *Y. enterocolitica* is a disease of middle aged and

elderly men (Plotkin and O'Rourke, 1981; Tame et al., 1998) who in most cases have bacteremia without any of the above risk factors for systemic yersiniosis. *Yersinia enterocolitica* (particularly serogroup O:9; La Scola et al., 1997) may be a more common cause of mycotic aneurysm (Prentice et al., 1993) than other better-recognized vasculotropic bacteria (Buckels et al., 1985) such as *Salmonella* and *Brucella* species. Serodiagnostic crossreactions (Weynants et al., 1996) between *Y. enterocolitica* serogroup O:9 and *Brucella* may account for previous associations between mycotic aneurysms and *Brucella* species (La Scola et al., 1997). Multiple mycotic aneurysms due to *Y. enterocolitica* requiring successive operations in the same patient have been described (Donald et al., 1996). Endocarditis is also recognized (Appelbaum et al., 1983; Giamairellou et al., 1995) and has now been reported from a total of 12 patients in the literature (Brouqui and Raoult, 2001), with no obvious serotype association.

**Other Acute Extraintestinal Conditions** Encephalopathy has been associated with *Y. enterocolitica* O:3 infection in a 10-year-old girl (Berner et al., 1998). Cellulitis has been associated with septicemia (Righter, 1981). Osteomyelitis of the tibia has been reported in a patient with Gaucher's disease without associated bacteremia (Fisch et al., 1989), and osteomyelitis with meningitis has also been reported (Casey et al., 1987). *Yersinia enterocolitica* infection of a prosthetic hip 15 years after insertion has been reported (i.e., presumed hematogenous spread; Hansen et al., 1989).

**Blood Transfusion** Severe, often fatal reactions after the transfusion of blood contaminated with environmental bacteria were well-recognized risks in the early days of blood transfusion (Braude et al., 1952). Single-unit, closed collection systems greatly reduced environmental contamination of donated blood (Walter et al., 1957) but have not eliminated the risk of contamination with *Y. enterocolitica* (Wagner et al., 1994). Since the first description in 1975 from The Netherlands (Bruining and De Wilde-Beekhuizen, 1975), over 35 cases have been reported worldwide (Hogman and Engstrand, 1996b). Common features of most reports include the refrigerated storage of whole blood or red cell concentrates for over three weeks before transfusion and serological evidence of donor infection with *Y. enterocolitica* at the time of donation (Prentice, 1992b; Hogman and Engstrand, 1996b). Recipients develop fever, respiratory distress, hypotension, and in some cases disseminated intravascular coagulation (Tipple et al., 1990; Anonymous, 1997). On standard investigation of

the transfusion reaction, large numbers of *Y. enterocolitica* are found in the remains of the transfused unit of blood, and the organism is grown from the recipient's blood (Prentice, 1992b; Hogman and Engstrand, 1996b). Over 50% of such reactions prove fatal (Tipple et al., 1990; Prentice, 1992a; Anonymous, 1997).

Experimental inoculation of small numbers of *Y. enterocolitica* (10–100 colony forming units [cfu]/ml) into units of whole blood (Stenhouse and Milner, 1982; Pietersz et al., 1992; Gibb et al., 1994) or red cell units (Arduino et al., 1989; Pietersz et al., 1992) stored at 4°C show a growth curve that passes through a lag phase lasting 7–10 days before a phase of exponential growth that after 2–3 weeks yields a concentration of 10<sup>8</sup> cfu/ml. This proliferation is accompanied by the presence in the red cell unit of endotoxin levels that are fatal in animal models (Arduino et al., 1989; Hastings et al., 1994; Anonymous, 1997). Transfusion of such a contaminated unit would produce severe shock as seen in the clinical case reports. The presumed mechanism of introduction of the organism to the unit is a low-grade bacteremia in the donor at the time of donation (Tipple et al., 1990; Hogman and Engstrand, 1996b). A key property is clearly that *Y. enterocolitica* is a psychrotroph: optimal growth temperature is above 20°C (i.e., a mesophile) but capable of growth at 4°C. Although (in general) platelets are far more commonly contaminated with bacteria than other blood components (1 in 2000 units of platelets is bacterially contaminated; Wagner et al., 1994), they are stored at 20–25°C with a shorter conservation period and there has only been one reported case of *Y. enterocolitica* sepsis following platelet transfusion (Kuehnert et al., 1997).

Experiments also suggest a role for complement in restricting *Y. enterocolitica* growth in the lag phase (Gibb et al., 1994) and in eliminating small inocula of *Y. enterocolitica* (<10<sup>2</sup> cfu/ml) from donated blood (Pietersz et al., 1992). This could be maximized by leaving units of whole blood at 18–24°C for at least 6 hours after donation before further fractionation and refrigeration (Pietersz et al., 1992) because this temperature (unlike 4°C) allows complement to be active and renders *Y. enterocolitica* serum-sensitive (Gibb et al., 1994) owing to a decrease in expression of plasmid genes induced at 37°C (Chiesa and Bottone, 1983). Unfortunately these conditions promote the growth of *Pseudomonas fluorescens* (Gibb et al., 1995), another psychrotrophic organism, which is a leading cause of bacterial contamination of blood products (Wagner et al., 1994). White cells have been shown to remove inoculated bacteria from donor blood (Hogman et al., 1991), presumably by phagocytosis. Because phagocytic cells lyse after

14 days storage (Hogman et al., 1992), phagocytosed but intracellularly surviving bacteria could contribute to appearance of *Y. enterocolitica* in donated blood after this time (Hogman et al., 1992). A role for white cells in *Y. enterocolitica* survival is suggested by the ability of white blood cell filtration after incubation at 22°C to remove inoculated *Y. enterocolitica* from stored blood (Hogman et al., 1992; Pietersz et al., 1992) and enhanced growth of *Y. enterocolitica* seen on inoculation into leucodepleted blood (Hogman et al., 1992).

It has been suggested (Gibb et al., 1996) that the widespread introduction of additive solutions, which prolong the viability of red cells, into blood transfusions in the 1980s was associated with the emergence of this phenomenon. This could have had indirect effects by allowing prolongation of storage well below the observed lag phase and direct effects because of reduced complement-mediated killing of contaminating *Y. enterocolitica* in additive-containing blood (Gibb et al., 1996). This hypothesis is disputed (Hogman, 1996a). The lack of reports of *Y. enterocolitica* serovar O:8 in transfusion-related incidents has been linked (Bottone, 1997) to more rapid loss of complement resistance at lower temperatures in this bioserovar compared to other pathogenic bioserovars of *Y. enterocolitica* (Chiesa and Bottone, 1983). The absence of reports of transfusion-related *Y. pseudotuberculosis* sepsis may be similarly related to differences in complement resistance. In this organism, unlike *Y. enterocolitica*, serum resistance is not a plasmid-dependent property (Perry and Brubaker, 1983). However, *Y. enterocolitica* 8081 (a serovar O:8 strain) and *Y. pseudotuberculosis* strains of serovar II, III and V have been shown to grow well at 4°C in units of whole blood for transfusion (Malbrunot and Guiyoule, 1990). Other factors, e.g., less prevalent infection with these organisms or less frequent asymptomatic bacteremia, could account for the paucity of transfusion reactions observed.

Control measures have been difficult to devise and cases continue (Strobel et al., 2000) despite widespread recognition of the problem. Techniques such as autologous transfusion that prevent transmission of other blood-borne infections do not prevent this syndrome (Sire et al., 1993; Haditsch et al., 1994). Donor exclusion by screening for recent gastrointestinal symptoms would exclude up to 6% of donors (Grossman et al., 1991) and would impose an unacceptable restriction on the blood supply without excluding risk from asymptomatic donors (McDonald et al., 1996). Reduction in the storage period of whole blood and red cells to less than 25 days would have implications for the blood supply (Hoppe, 1992) and would not eliminate risk.

Individual testing of units immediately after collection, in parallel with other tests for blood-borne diseases, would require very sensitive assays to detect the small numbers of organisms capable of causing the problem, and testing immediately prior to transfusion would mean introducing an extra step in the transfusion process. Either of these strategies would have major cost and logistic implications. A recent international symposium was unable to suggest a simple method for the elimination of this problem (Klein et al., 1997). There is no international consensus (Reesink et al., 1993) on measures to maximize bactericidal action of whole blood as indicated above. Currently some countries (e.g., the Netherlands and Ireland) practice rapid cooling of donated whole blood to 20–22°C followed by an overnight hold at that temperature before component separation and leucocyte filtration (Reesink et al., 1993). Other countries (e.g., the United Kingdom and United States) do not have this as national policy (Reesink et al., 1993). There have been no cases reported via the (<http://www.shot.demon.co.uk>{Serious Hazards of Transfusion scheme}) in the United Kingdom since leucodepletion of all units commenced in November 1999.

A major factor complicating development of preventive measures has been the rarity of the syndrome. Most effort in finding and reporting cases has been in the United States where an estimate of one *Yersinia*-associated RBC transfusion reaction per 500,000 units has been made (United States General Accounting Office, 1997). Other countries have reported much higher rates, e.g., 1 in 65,000 in New Zealand (Theakston et al., 1997) where *Y. enterocolitica* is a leading enteric pathogen (Fenwick and McCarthy, 1995). Concern that under-reporting of *Y. enterocolitica* and other bacteria-associated blood transfusion reactions was occurring in the United States led to a prospective study organized by the United States Centers for Disease Control Hospital Infections Program (Anonymous, 1997). The data from BaCon Study (Assessment of the Frequency of Blood Component Bacterial Contamination Associated Transfusion Reaction), conducted from 1997–2000, are currently being analyzed.

**POST-INFECTIVE PHENOMENA** Reactive arthritis is a sterile, usually self-limiting arthritis following a gastrointestinal, urogenital or pharyngeal infection (Sieper and Braun, 1999a; Immunology, paragraph reactive arthritis) often accompanied by other complications such as erythema nodosum, keratoderma, uveitis or conjunctivitis (Gaston, 2001). Typically, an asymmetric arthritis of the lower limbs is seen, and 50% of the patients are human leukocyte antigen (HLA)-



B27 positive (Sieper et al., 1996). Because of some shared symptoms, pathology, and probable post-infective etiology, reactive arthritis is grouped together with inflammatory bowel disease associated arthritis, psoriatic arthritis, undifferentiated arthritis and ankylosing spondylitis as a spondyloarthropathy (arthropathy affecting the axial skeleton; Gaston, 2001) even though in reactive arthritis the axial skeleton and sacroiliac joints are generally less commonly involved than the lower limbs (Gaston, 2001).

*Yersinia enterocolitica* is one of the most common microorganisms associated with reactive arthritis (Valtonen et al., 1985) or undifferentiated arthritis (a similar clinical picture where there is no obvious prior infection; Van der Heijden et al., 1997; Fendler et al., 2001) and is linked with 18–25% of cases in these series. This association is supported by detection of high titers of circulating IgA and IgG antibodies to *Y. enterocolitica* and *Y. pseudotuberculosis* (Fendler et al., 2001), detection of *Y. enterocolitica* antigens in colonic and synovial tissue (Hoogkamp-Korstanje et al., 1988; Granfors et al., 1989; Van der Heijden et al., 1997), and cloning of *Y. enterocolitica*-specific T-cells from the synovial tissue (Van der Heijden et al., 1997) of patients with reactive or undifferentiated arthritis. Culture or detection of *Y. enterocolitica* DNA by PCR in synovial tissues in reactive or undifferentiated arthritis has been mainly negative (Nikkari et al., 1992; Braun et al., 1997) despite the repeated detection of *Y. enterocolitica* antigens in these tissues (Viitanen et al., 1991; Nikkari et al., 1992; Van der Heijden et al., 1997). The PCR amplification of a 16S rRNA gene segment with a closest fit to *Y. enterocolitica* has been reported from synovial fluid in one patient with arthropathy (Wilkinson et al., 1999).

A consensus view (Sieper et al., 2000) is that the presence of living bacteria in synovium is not a requirement for reactive arthritis, but the continuing presence of bacterial antigens in the joint and in circulating white cells (Granfors et al., 1998) is part of the process. The precise relationship between activated synovial or circulating T cells specific for bacterial antigens, host HLA-B27 status, and the disease process is not clear (Sieper et al., 2000; Gaston, 2001). The absence of an active infection with viable bacteria accords with the lack of improvement over placebo shown in controlled trials of ciprofloxacin therapy for reactive arthritis due to *Yersinia* (Sieper et al., 1999b) or reactive arthritis in general (Toivanen et al., 1993; Sieper et al., 1999b). However, antibiotic therapy has been useful in chronic relapsing gastrointestinal disease caused by *Y. enterocolitica* (Hoogkamp-Korstanje et al., 1988) and in one reactive arthritis trial, ciprofloxacin eliminated *Y. enterocolitica* antigen from

colonic biopsies and achieved a nonsignificant trend to more rapid remission than placebo (Hoogkamp-Korstanje et al., 2000).

Raised antibody titers to *Y. enterocolitica* are seen in patients with autoimmune thyroid disorders (Bech et al., 1974), partly due to thyrotrophin (TSH) binding sites on *Y. enterocolitica* recognized by these autoantibodies (Heyma et al., 1986). It is not thought that *Y. enterocolitica* infection is a major factor in triggering thyroid disease (Toivanen and Toivanen, 1994).

### Bacteriological Diagnosis of *Y. enterocolitica* (Georges Wauters)

Non-pestis yersiniae include two pathogenic species: *Y. pseudotuberculosis*, genetically related to *Y. pestis*, and *Y. enterocolitica*. Besides the latter species, several strains are known as “*enterocolitica*-like organisms” and were later assigned to the so-called “related species”: *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. aldovae*, *Y. rohdei*, *Y. mollaretii* and *Y. bercovieri*. *Yersinia ruckeri* has been tentatively included in the genus *Yersinia*, but its taxonomic position is controversial (Taxonomy).

Non-pestis *Yersinia* strains exhibit the general characteristics of Enterobacteriaceae, being fermentative, oxidase-negative, Gram-negative rods and growing on ordinary media. Except for *Y. ruckeri*, common features of these species are the presence of urease, with very few exceptions. They lack lysine decarboxylase, arginine dihydrolase, phenylalanine deaminase and do not produce H<sub>2</sub>S on Kligler agar.

They have also common characteristics with regard to growth temperature. Optimal growth occurs at 30–32°C rather than 37°C. However, some properties (like motility and acetoin production) are only detected when the incubation temperature is below 30°C. Other biochemical features (like urease and some assimilation and fermentation reactions) may also be weak and delayed, or even negative at 37°C. These organisms are psychrophilic and are able to grow, although slowly, at 4°C. Conversely, in pathogenic species, most virulence factors are expressed only at 37°C. The choice of the incubation temperature therefore depends on the purpose of the bacteriological investigation.

ISOLATION OF *Y. ENTEROCOLITICA* *Yersinia enterocolitica* can be recovered on some selective media currently used for stool culture, e.g., Salmonella-Shigella (SS) and deoxycholate (DCL) agar, but colonies are small and when incubated at 37°C, require 48 h for optimal detection. A better way is to incubate overnight at 37°C followed by an additional 24 h at room temperature. Media containing sucrose such as

deoxycholate citrate lactose sucrose (DCLS) agar and Hektoen agar should not be used since *Y. enterocolitica* acidifies sucrose hindering any detection of suspect colonies. More specific isolation media may be considered:

CIN (cefsulodin, irgasan, and novobiocin) agar is widely used for isolation of the organism from feces and food (Schiemann, 1979). Optimal incubation temperature should be 30–32°C, but 37°C and room temperature are also suitable. Colonies are quite typical after 24 h of incubation at 30°C; they have a “bull’s eye” appearance with a small red center surrounded by a colorless area. After 48 h, they are larger, the red center becomes diffuse, and they are readily mistaken for other enterobacteria, e.g., *Citrobacter*. Therefore, prolonged incubation of CIN at 30°C is of little value.

SSDC (SS agar supplemented with 1% deoxycholate and 0.1% of CaCl<sub>2</sub>; Wauters et al., 1988a) must be incubated at 30–32°C for 24–48 h, and not at 37°C. Colonies are small and colorless after 24 h of incubation, but since the medium is highly inhibitory, colonies of other bacteria may be even smaller. This medium is mainly useful when combined with an enrichment method.

**ENRICHMENT PROCEDURES** Cold enrichment was first advocated to increase the isolation rate of *Y. enterocolitica* from feces and food. Specimens are suspended in phosphate buffer pH 7.6 and allowed to stay 1–3 weeks at 4°C (Ahvonen, 1972; Eiss, 1975; Toma and Deidrick, 1975). A subculture is made onto CIN or SSDC agar, with or without a KOH treatment of the inoculum, as described by Aulisio et al. (1980). For feces, however, this method is not very useful because of the long delay. Moreover, cold enrichment mainly improves the recovery of nonpathogenic *Yersinia* biotypes and species (Van Noyen et al., 1980).

ITC (irgasan, ticarcilin, and chlorate) broth incubated for 2 days at 24°C results in a significant increase of biotype 4 (serotype 3) strains, but is not suitable for other bioserotypes. Use of SSDC rather than CIN agar is recommended for subculturing from ITC broth. The usefulness of this enrichment is particularly obvious in the detection of *Yersinia* in food (Wauters et al., 1988a).

**IDENTIFICATION** Any colony displaying presumptive characteristics of *Yersinia* should be carefully and completely identified, since biotypes of *Y. enterocolitica* may have a different pathogenic relevance and some related species may closely resemble *Y. enterocolitica* sensu stricto. The main differential characteristics of *Yersinia* species are reported in Table 1. Four key-tests allow the dif-

ferentiation of *Y. enterocolitica* sensu stricto from related species. *Yersinia enterocolitica* is sucrose positive and rhamnase negative and does not assimilate citrate or mucate. At least one of these tests gives an opposite result in the other species.

Commercial systems like API 20E and ID 32E usually achieve a reliable identification of *Y. enterocolitica*, but to a lesser extent of the related species.

*Yersinia enterocolitica* strains have been classified into five biotypes, the first of which is subdivided into biotypes 1<sup>A</sup> and 1<sup>B</sup>. The biotype scheme is based on the following properties: Tween esterase, rapid esculin hydrolysis (less than 24 h), pyrazinamidase, indole production, acid from xylose and trehalose, and nitrate reduction (Wauters et al., 1987; Table 2).

More than 75 somatic antigens (O-antigens) have been described in *Y. enterocolitica* and the related species (Wauters et al., 1991). There are also a large number of flagellar antigens that are not currently used in antigenic typing. Different *Yersinia* species and biotypes may share common O-antigens. In *Y. enterocolitica*, there is some correlation between biotype, antigenic pattern, and ecological behavior. Strains that are pathogenic for man or animals belong to biotypes 1<sup>B</sup>, 2, 3, 4 and 5 and possess only a limited number of O-factors. Biotype 1<sup>B</sup> includes strains mainly possessing antigens O:8, O:13 and O:21. Biotype 2 consists of strains possessing antigens O:9 (which share antigenicity with *Brucella*) and O:5,27. Biotype 3 includes factor O:1,2,3 and more recently the Voges-Proskauer (VP)-negative Asiatic O:3 strains. In biotype 4, only serotype O:3 is found (the most widespread human pathogen), and in biotype 5, only serotype O:2,3 (in animals). Strains of biotype 1<sup>A</sup> are nonpathogenic, ubiquitous, and are often found in food and in the environment. Hence, they may be occasionally encountered in the digestive tract of animals and humans. They carry a large variety of O-antigens of which O:5, O:6 and O:7,8 are the most common. Although some O antigens like O:3, O:9 or O:8 are regularly found in pathogenic strains, they should not be considered as specific since they sometimes occur in other species or nonpathogenic biotypes, e.g., O:8 in *Y. bercovieri* or in biotype 1<sup>A</sup>, O:9 in *Y. frederiksenii*, O:1,2,3 in biotype 1<sup>A</sup>, O:3 in *Y. mollaretii*, etc. Therefore, serotyping should not be performed as a single procedure on suspected isolates but only after appropriate species and biotype determination.

**VIRULENCE AND PATHOGENICITY MARKERS OF *Y. ENTEROCOLITICA*** The pYV virulence plasmid of *Y. enterocolitica* plays an important part in the pathogenicity of the organism and encodes

Table 1. Biochemical characteristics of *Yersinia* species.<sup>a</sup>

	URE	ODC	PYZ	IND	VP	CIT	MUC	T80	ESC	SUC	RHA	MEL	AMG	CEL	SBT	SBS	FUC
<i>Y. pestis</i>	-	-	-	-	-	-	-	-	+	-	-	v	-	-	-	-	nd
<i>Y. pseudotuberculosis</i>	+	-	-	-	-	-(+)	-	-	+	-	+	+/-	-	-	-	-	-
<i>Y. enterocolitica</i>	+	+ <sup>b</sup>	v	v	+/-	-	-	v	v	+ <sup>b</sup>	-	-	-	+	+/-	v	v
<i>Y. intermedia</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v
<i>Y. frederiksenii</i>	+	+	+	+	+/-	+/-	+	v	+	+	+	-	-	+	+	+	+
<i>Y. kristensenii</i>	+	+	+	v	-	-	v	v	v	-	-	-	-	+	+	+	v
<i>Y. rohdei</i>	+	+	+	-	-	+	-	-	-	+	-	v	-	+	+	nd	nd
<i>Y. aldovae</i>	+	+	+	-	+	+	v	v	-	-	+	-	-	-	+	-	v
<i>Y. bercovieri</i>	+	+	+	-	-	-	+	-	v	+	-	-	-	+	+	-	+
<i>Y. mollaretii</i>	+	+	+	-	-	-	+	-	v	+	-	-	-	+	+	+	-
<i>Y. ruckeri</i>	-	+	nd	-	-	-	-	nd	nd	-	-	-	-	-	-	nd	nd

Symbols: +, positive; -, negative; +/-, most strains positive; -/+, most strains negative; v, variable; (+), weak reaction; and nd, not done.

Abbreviations: URE, urease; ODC, ornithine decarboxylase; PYZ, pyrazinamidase; IND, indole; VP, Voges-Proskauer; CIT, Simmons' citrate; MUC, mucate; T80, Tween esterase; ESC, esculin; SUC, acid from sucrose; RHA, acid from rhamnose; MEL, acid from melibiose; AMG, acid from  $\alpha$ -methyl-D-glucoside; CEL, acid from cellobiose; SBT, acid from sorbitol; SBS, acid from sorbose; and FUC, acid from fucose.

<sup>a</sup>Tests are performed at 25°C.

<sup>b</sup>Some strains of biotype 5 are negative.

Table 2. Biotypes of *Yersinia enterocolitica*.

	1 <sup>A</sup>	1 <sup>B</sup>	2	3	4	5
Esculin (<24h)	+	-	-	-	-	-
Pyrazinamidase	+	-	-	-	-	-
Tween esterase	+	+	-	-	-	-
Indole	+	+	(+)	-	-	-
Xylose	+	+	+	+	-	v
Trehalose	+	+	+	+	+	-
NO <sub>3</sub>	+	+	+	+	+	-

Symbols: +, positive; (+), weak or delayed positive; -, negative; and v, variable.

several properties that can be detected by in vitro tests (pYV virulence plasmid). Some methods for detecting pYV are easy to perform routinely and most are correlated with the production of the YadA protein. The following are the most useful:

Calcium dependency at 37°C can be detected on Congo red-magnesium oxalate (CR-MOX) agar (Riley and Toma, 1989). Plasmid-bearing bacteria produce small red colonies.

Autoagglutination of pYV<sup>+</sup> strains in broth at 37°C. Although the test is very simple, it is not as reliable as others.

Agglutination of the strain grown on tryptic soy agar at 37°C by the phytoagglutinins of *Mangifera indica* (Wauters et al., 1995) or *Persea americana*.

Agglutination of the strain grown on tryptic soy agar by an antiserum against the YadA protein, which is not commercially available (Sory et al., 1990).

The pYV plasmid can be lost after a few subcultures, and it might be important to assess the potential pathogenicity of a strain, even if plasmidless.

Two biochemical tests, pyrazinamidase and rapid esculin hydrolysis, may offer a screening method in this respect. Strains that are pyrazinamidase and/or esculin positive are likely to be nonpathogenic (biotype 1<sup>A</sup> and related species). Strains that are pyrazinamidase and esculin negative are likely to be pathogenic (biotype 1<sup>B</sup>, 2, 3, 4 and 5).

Moreover, potential pathogenic strains, even after losing the virulence plasmid, produce at 37°C, but only on acid media, a fibrillar protein called “mucoid *Yersinia* factor” or Myf (Other putative or proven virulence factors). It can be detected in strains grown at low pH and 37°C by an agglutination test using an anti-Myf serum (Leiva et al., 1995).

Recently Fenwick et al. described *Y. enterocolitica*-like strains with atypical characteristics such as a negative ornithine or acid production from melibiose. They possess two new somatic antigens designated “O:77” and “O:78.” They

exhibit a borderline genomic relationship with *Y. enterocolitica*. Virulence markers are negative and no pathogenicity has been found. Up to now, these strains were only isolated in New Zealand (Fenwick et al., 1996).

**DIFFERENTIATION OF *Y. enterocolitica* FROM RELATED *Yersinia* SPECIES** These species (like *Y. enterocolitica* biotype 1<sup>A</sup>) are frequently isolated from food and are also found as transient organisms in the digestive tract of humans or animals. They should be carefully differentiated from *Y. enterocolitica sensu stricto* (Bercovier et al., 1980a), especially from the pathogenic strains, since the related species have no or little clinical relevance. Out of more than 200 strains isolated from human stools, *Y. frederiksenii* and *Y. bercovieri* together accounted for 76% of the isolates; the remaining 24% were *Y. kristensenii*, *Y. intermedia*, *Y. mollaretii* and *Y. rohdei*.

*Y. intermedia* This species has been described by Brenner et al. (1980a) and displays strong metabolic activity: many carbohydrates (e.g., rhamnose, melibiose, raffinose, and  $\alpha$ -methylglucoside) are acidified, and Simmons' citrate is positive (Table 1). However, some variability is observed in these tests, allowing 8 biotypes to be identified within the species (Brenner et al., 1980a). Except for some rare isolates from stools, a few strains have been recovered from extra-intestinal sites in humans (Bottone, 1978; Farmer et al., 1985).

*Y. frederiksenii* *Yersinia frederiksenii* also has been described in 1980 by Ursing (Ursing et al., 1980), but this species is genetically heterogeneous, and phenotypic characteristics are also quite variable. *Yersinia frederiksenii* (unlike *Y. intermedia*) strains do not ferment melibiose and  $\alpha$ -methylglucoside. Citrate and Tween-esterase give different results (Table 1). This is one of the related species most frequently encountered in humans.

*Y. kristensenii* The description of this species was done by Bercovier et al. in 1980 (Bercovier et al., 1980c). *Yersinia kristensenii* has few positive biochemical properties, ferments neither sucrose nor many other sugars, and never produces acetoin. The positive ornithine reaction and the lack of rhamnose acidification help avoid confusion with *Y. pseudotuberculosis*. Pyrazinamidase is often weak in this species, hampering the screening for pathogenic strains.

*Y. aldovae* Described in 1984 by Bercovier et al. (1984), this species, which is rhamnose positive but sucrose negative, has never been isolated in humans.

*Y. rohdei* Aleksic et al. described in 1987 (Aleksic et al., 1987) a new species, which is mucate negative like *Y. enterocolitica*, but citrate positive unlike the latter.

*Y. mollaretii* and *Y. bercovieri* These closely related species were described in 1988 by Wauters et al. (1988b). Acid production from fucose and sorbose are the only differences between the two species (Table 1). Among the related species, *Y. bercovieri* and *Y. mollaretii* are most similar to *Y. enterocolitica*. The main difference is the ability to assimilate mucate, which is not present in *Y. enterocolitica* sensu stricto. They can also be differentiated from the pathogenic *Y. enterocolitica* biotypes by a positive pyrazinamidase test. Another important feature is the lack of acetoin production, which is as a rule positive in pathogenic *Y. enterocolitica*, except in the VP negative variant of biotype 3.

It should be noted that *Y. bercovieri* is the second most prevalent *Yersinia* species in humans among the related species, just after *Y. frederiksenii*. It is frequently isolated from food as well. Most commercial identification systems do not include these species in their database and record them as *Y. enterocolitica*.

*Y. ruckeri* This fish pathogen (redmouth bacterium) has been included in the genus *Yersinia* by Ewing in 1989 (Ewing et al., 1978), but this inclusion is controversial (De Grandis et al., 1988a). The biochemical reactions of *Y. ruckeri* do not fit the general profile of *Yersinia* and some strains may be lysine and gelatin positive. Growth at 37°C is poor. The only report of a human isolate of *Y. ruckeri* is not fully documented and may be questionable (Richard, 1984).

## ***Yersinia pseudotuberculosis***

### Epidemiology of *Y. pseudotuberculosis* (Hiroshi Fukushima)

**HUMAN INFECTIONS** *Yersinia pseudotuberculosis* has a wide distribution in most countries with cold climates and is recognized as an important causative agent of sporadic and epidemic human enteric diseases (Mair, 1965; Tsubokura et al., 1989; Table 3).

The main clinical manifestations of human infections in Europe (Clinical manifestations of *Y. pseudotuberculosis* infections) are fever and gastroenteritis (Winblad, 1967; Ahvonen, 1972; Aleksic et al., 1995; Table 4). Septicemic forms of *Y. pseudotuberculosis* infections are rare, but are frequent in patients with underlying disorders such as diabetes, hepatic cirrhosis or iron-overload (Ljungberg et al., 1995; Table 5).

In contrast, the clinical features of human infections in Japan (Sato et al., 1983; Fukushima et al., 1985b; Tsubokura et al., 1987), Far East Russia (Somov and Martinevsky, 1973), and Korea (Cheong et al., 1995) are not only gastrointestinal symptoms but also a variety of systemic manifestations such as fever, scarlatina-like rash, desquamation, erythema nodosum, and arthritis (Table 6 and Fig. 1). The major differences in clinical symptoms between Far Eastern and Western countries are rash and desquamation, which are not seen in Western patients but are common in the Far East. Moreover, in Japan approximately 10% of the infected children fulfill diagnostic criteria of Kawasaki disease, including coronary vasculitis (Baba et al., 1991). Acute renal failure, mostly tubulointerstitial nephritis, is also seen in these patients (Takeda et al., 1991; Cheong et al., 1995).

It appears that the difference in clinical manifestations of *Y. pseudotuberculosis* infection between the Far Eastern and Western countries is related to the heterogeneity in the distribution of *Yersinia pseudotuberculosis* superantigen (YPMa) and high-pathogenicity island (HPI).

In sporadic cases, the highest incidence of infection is in 1–5 year-old children, with a peak at 2 years of age (Sato, 1987a; Aleksic et al., 1995; Fig. 2).

The female : male ratio is 1 : 1 in Germany and Finland (Ahvonen, 1972; Aleksic et al., 1995) but 1:1.5 in Japan (Tsubokura et al., 1987).

The highest incidence is observed during the coldest months, regardless of the geographical areas (Mair, 1965; Fukushima et al., 1985b; Sato, 1987a; Tsubokura et al., 1987; Aleksic et al., 1995; Fig. 3).

Although small outbreaks attributable to *Y. pseudotuberculosis* in some families have been documented in Europe (Aleksic et al., 1995), large scale outbreaks of *Y. pseudotuberculosis* occurred mainly through consumption of vegetables or water contaminated with this organism in Japan (Inoue et al., 1984a; Fukumoto et al., 1987; Sanbe et al., 1987; Inoue et al., 1988; Nakano et al., 1989; Tsubokura et al., 1989; Toyokawa et al., 1993) and Far East Russia (Shiozawa et al., 1988; Fukushima et al., 1998b), and in Finland (Tertti et al., 1984; Table 7).

### DISTRIBUTION OF THE SUBGROUPS OF *Y. PSEUDOTUBERCULOSIS*

**Serotypes of *Y. pseudotuberculosis*** There are 21 different serotypes of *Y. pseudotuberculosis*: O:1a, 1b, 1c, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 (Tsubokura and Aleksic, 1995; Nagano et al., 1996; Table 8).

In Western countries (Europe, Australasia and North America; Mair, 1965; Hubbert, 1972;

Table 3. Reports of isolation of *Y. pseudotuberculosis* from human infections.

Countries	No. of samples	No. of positive	Isolation rates	No. of strains	Serotypes																	References												
					1	1a	1b	1c	2	2a	2b	2c	3	3 NMF <sup>a</sup>	4	4a	4b	5	5a	5b	6		7	9	10	11	12	13	14	15	UT	Year		
England	0	0	0	8	0	5	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1961–1964	Mair, 1965
Germany	0	0	0	54	0	21	10	0	0	7	3	0	1	6	0	0	0	2	0	2	0	0	0	0	0	0	0	0	0	2	1983–1993	Aleksic et al., 1995		
The United States	0	0	0	13	0	+	+	0	0	0	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–1972	Hubbert, 1972	
Canada	0	0	0	16	0	0	10	0	0	0	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1962–1985	Toma, 1986	
Italy	9720	5	0.05	5	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1981–1991	Chiesa et al., 1993	
Japan	9820	33	0.3	33	0	0	12	0	0	0	1	0	2	0	0	0	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1976–1986	Fukushima et al., 1985b	
Japan	0	0	0	260	0	0	6	0	0	9	20	10	6	0	0	7	121	0	33	48	0	0	0	0	0	0	0	0	0	0	0	1979–1989	Sato and Komazawa, 1991	
Korea	0	0	0	19	0	1	2	0	0	2	0	0	3	0	0	1	7	0	0	3	0	0	0	0	0	0	0	0	0	0	0	1987–1995	Cheong et al., 1995	
Korea	0	0	0	48	0	0	0	0	0	0	0	0	0	0	0	0	18	0	0	0	0	0	0	0	0	0	0	0	0	30	1993–1996	Fukushima et al., 1998		
Russia	0	0	0	45	0	0	20	0	0	0	0	0	7	0	0	1	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1975–1995	Fukushima et al., 1998	

Abbreviations: UT, untyped; and for other definitions, refer to footnote in Table 12.  
<sup>a</sup>NMF, non-melibiose fermenting strains.

Table 4. Clinical manifestations of human *Y. pseudotuberculosis* infections.

Clinical manifestations	No. of patients	
	Germany <sup>a</sup>	Sweden <sup>b</sup>
Acute terminal ileitis	0	14
Enteritis	41	16
Abdominal pain	2	10
Mesenteric lymphadenitis	2	15
Pseudoappendicitis	3	5
Sepsis	3	0
Erythema nodosum	0	10
Joint pain	2	0
Healthy	1	0
Total	54	70

<sup>a</sup>From Aleksic et al. (1995).

<sup>b</sup>From Winblad (1967).

Table 5. Underlying disorders in 54 patients with *Yersinia pseudotuberculosis* septicemia.

	No. of patients
Hemochromatosis/hemosiderosis <sup>a</sup>	9
Hepatic cirrhosis <sup>a</sup>	7
Diabetes	8
Alcohol abuse <sup>a</sup>	4
Chronic active hepatitis <sup>a</sup>	1
Sickle-cell anemia <sup>a</sup>	1
Amyloidosis	1
Universal arteriosclerosis	1
Thalassemia major <sup>a</sup>	1
Polycythemia vera <sup>a</sup>	1
Chronic intestinal nephritis and dialysis	1
Kidney transplant	1
Ventriculoperitoneal shunt	1
None found	17
Not known	12

<sup>a</sup>An iron overload disorder.

From Ljungberg et al. (1995).

Table 6. Clinical manifestations of *Y. pseudotuberculosis* in children.

Clinical manifestations	Percent of patients		
	Japan <sup>a</sup>	Korea <sup>b</sup>	Finland <sup>c</sup>
Fever >38°C	99	100	90
Recurrent fever	53	0	0
Rash	77	79	0
Erythema nodosum	20	0	47
Desquamation	83	58	0
Diarrhea, loose stools	68	0	23
Abdominal pain	96	84	67
Right lower quadrant tenderness	37	0	33
Hepatomegaly	18	0	0
Upper respiratory	41	0	0
Eye inflammation	43	0	3
Strawberry tongue	58	37	0
Redness of throat	30	0	0
Pneumonia	4	0	0
Cervical lymphadenopathy	31	0	0
Joint pain	47	16	10
Arthritis	1	26	10
Osteomyelitis	1	0	0
Acute renal failure	12	0	0
Leukocytosis	45	0	0
Elevated E.S.R.	63	100	0
Numbers of patients	164	11	70

Abbreviation: E.S.R., erythrocyte sedimentation rate.

<sup>a</sup>Sato (1987).

<sup>b</sup>Cheong et al. (1995).

<sup>c</sup>Ahvonon (1972).

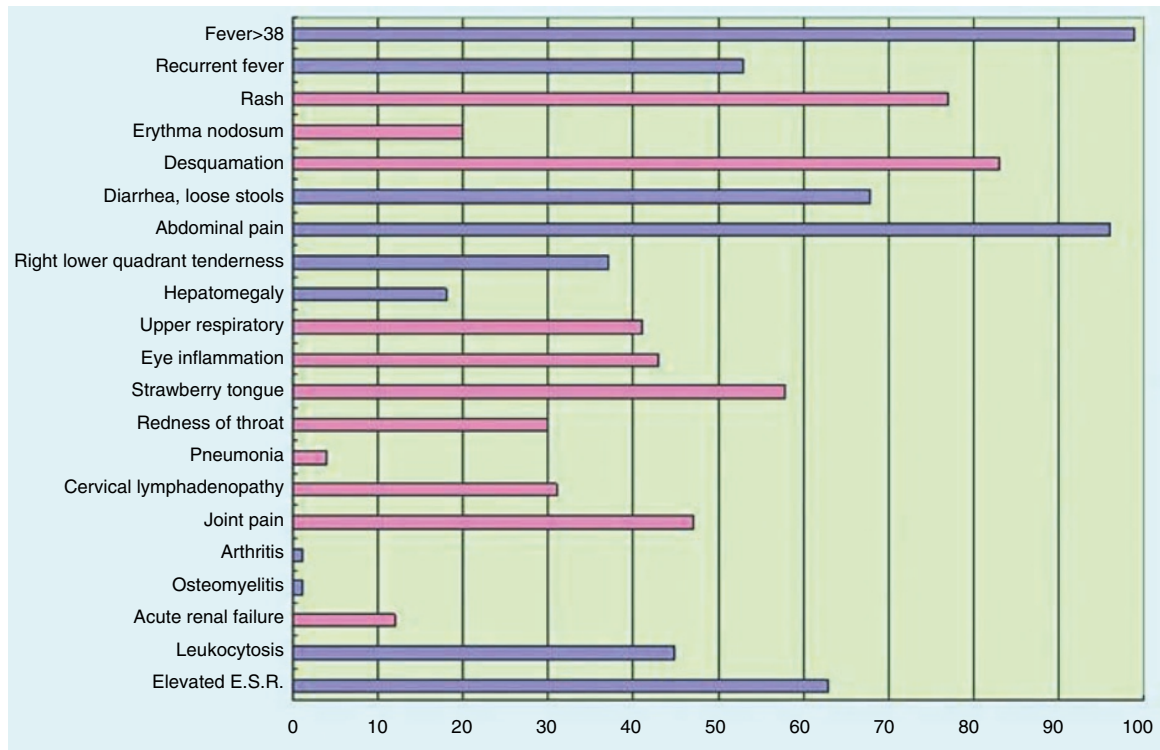


Fig. 1. Clinical manifestations in 164 Japanese children with *Yersinia pseudotuberculosis* infection. From Sato (1987b).

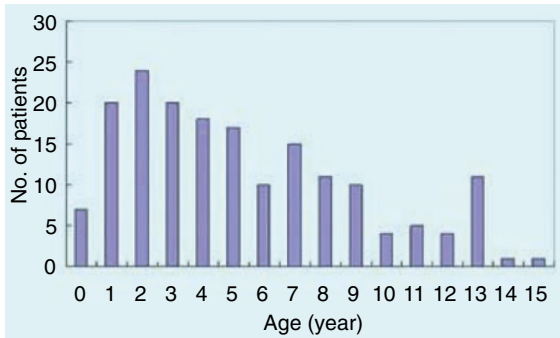


Fig. 2. Age distribution of *Yersinia pseudotuberculosis* infections. From Sato (1987a); Sato (1987b).

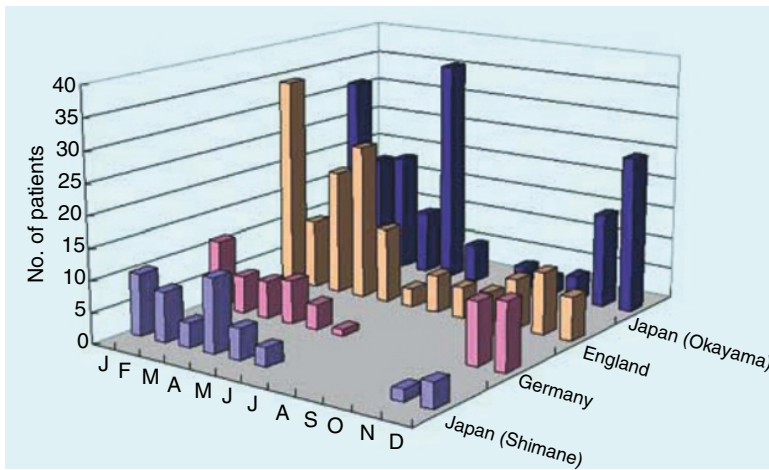


Fig. 3. Monthly occurrence of *Yersinia pseudotuberculosis* infections. From Sato (1987a), Sato (1987b), Fukushima et al. (1985c), Aleksic et al. (1995), and Mair (1963).

Table 7. Outbreaks of *Y. pseudotuberculosis* infection.

Country	Outbreak	Location	Suspicious materials	No. of patients	Diagnosed using	Serotype	References
Japan	Apr 1977 <sup>a</sup>	Middle school	Unknown	57	Antibody	5b	Tsubokura et al., 1989
	Oct 1977 <sup>a</sup>	Kindergarten	Water (?)	82	Antibody	1b	Tsubokura et al., 1989
	Feb 1981 <sup>a</sup>	Elementary school	Vegetable juice	188	Isolation	5a	Inoue et al., 1984
	Feb 1981	Urban	Sandwiches	11	Isolation	5b	Inoue et al., 1988
	Dec 1982	Mountain area	Stream water	140	Isolation	4b	Inoue et al., 1988
	July 1984 <sup>b</sup>	Middle school	Barbecue	35	Isolation	5a	Nakano et al., 1989
	July 1984 <sup>b</sup>	Family	Barbecue	4	Isolation	5a	Nakano et al., 1989
	Nov 1984	Elementary school and kindergarten	Unknown	63	Isolation	3	Tsubokura et al., 1989
	Nov 1984	Mountain area	Water	11	Isolation	4b	Inoue et al., 1988
	Apr 1985	Elementary school and kindergarten	Unknown	8	Isolation	4b	Tsubokura et al., 1989
	Apr 1985	Elementary school	Unknown	60	Isolation	4b	Tsubokura et al., 1989
	Mar 1986	Elementary school	School lunch	549	Isolation	4b	Sanbe et al., 1987
	May 1987	Mountain area	Well water	5	Isolation	3	Tsubokura et al., 1989
	May 1988	Mountain area	Spring water	31	Isolation	3	Tsubokura et al., 1989
	June 1991 <sup>a</sup>	Elementary and middle school	School lunch	732	Isolation	5a	Toyokawa et al., 1993



Table 7. *Continued*

Country	Outbreak	Location	Suspicious materials	No. of patients	Diagnosed using	Serotype	References
Russia	1959	Urban	Vegetable (?)	300	Isolation	Unknown	Somov and Martinevsky, 1973
	Mar 1971	Urban	Vegetable (?)	826	Isolation	Unknown	Somov and Martinevsky, 1973
	1971	Urban (four outbreaks)	Vegetable (?)	Unknown	Isolation	Unknown	Somov and Martinevsky, 1973
	July 1983	Urban	Vegetable	Unknown	Isolation	1b	Fukushima et al., 1998
	Apr 1985	Urban	Vegetable	Unknown	Isolation	4b	Fukushima et al., 1998
	July 1987	Settlement	Vegetable	Unknown	Isolation	1b	Fukushima et al., 1998
Finland	Dec 1981–Feb 1982	Urban	Unknown	19	Isolation and antibody	3	Tertti et al., 1984

<sup>a</sup>Diagnosed as Izumi fever.

<sup>b</sup>Same restaurant.

Table 8. Antigenic scheme for *Y. pseudotuberculosis*.

O groups	O subgroups	O antigens	H antigens
1	1a	2, 3, 23	a, c
	1b	2, 4, 23	a, c
	1c	2, 4, 17, 24	b, c, d
2	2a	5, 6, 16	a, d
	2b	5, 7, 16, 17	a, d
	2c	5, 7, 11, 18	a, d
3		8, 15	a
4	4a	9, 11	b: a, b
	4b	9, 12	a, b, d
5	5a	10, 14, 32	a: a, e (b)
	5b	10, 15, 33	a
6		13, 19, 26	a
7		19, 13	a
8		20	a
9		10, 25	a, b, d
10		26	a, d
11		27, 4, 14, 15	b, d
12		28	a, d
13		29	—
14		30	a, b, d
15		31, 2, 10, 32, 33	—

Symbol: —, H-antigen absent.

From Tsubokura and Aleksic (1995) and Nagano et al. (1997).

Bissett, 1979; Toma, 1986; Aleksic et al., 1995; Fig. 4), serotypes O:1a, 1b, 2a, 2b, 3, 5a and 6, of which serotypes O1a and 1b are dominant, have been isolated from clinical samples (Table 3).

In the Far East (Fukushima et al., 1985b; Fukushima et al., 1987; Tsubokura et al., 1989; Sato and Komazawa, 1991; Cheong et al., 1995; Fukushima and Gomyoda, 1995a), serotypes O1b, 2a, 2b, 3, 4a, 4b, 5a, 5b and 15, of which serotypes O4b and 5b are dominant, have been isolated from clinical samples (Tables 3 and 9).

In the Far East, serotypes O:7 to O:14 have been isolated only from wild animals and the

environment but never from clinical samples (Fukushima et al., 1990a; Fukushima et al., 1995c; Fukushima and Gomyoda, 1991b; Zheng et al., 1995; Nagano et al., 1997b).

The pathogenicity of *Y. pseudotuberculosis* depends on the presence of a 70-kb virulence plasmid termed “pYV” (Gemski et al., 1980). This plasmid is essential for virulence and its presence differentiates pathogenic from non-pathogenic *Yersinia* (pYV virulence plasmid). The pYV is found in almost all serotypes O:1a to 6, 10 and 15 but not in serotypes O:7, 8, 9, 11, 12, 13 and 14 (Fukushima et al., 1990a; Fukushima et al., 2001b; Kaneko et al., 1991; Fukushima and Gomyoda, 1995b; Zheng et al., 1995; Nagano et al., 1996). Additionally, a pathogenicity island termed “high-pathogenicity island” (HPI; Carniel, 1999; High Pathogenicity Island) and/or chromosomally encoded novel superantigenic toxins designated “*Y. pseudotuberculosis*-derived mitogen”: YPMa (Abe et al., 1993; Uchiyama et al., 1993; Ito et al., 1995; Yoshino et al., 1995a; Ueshiba et al., 1998; Carnoy et al., 2000), YPMb (Ramamurthy et al., 1997a), or YPMc (Carnoy and Simonet, 1999) are found in *Y. pseudotuberculosis* (Superantigens of *Y. pseudotuberculosis*).

#### *Genetic Groups of Y. pseudotuberculosis and Their Geographical Distribution*

The relationship between the presence of YPM and HPI in *Y. pseudotuberculosis* in 2235 strains collected all over the world (Fukushima et al., 2001b; Table 10 and Figs. 4–6), and restriction endonuclease analysis of pYV (REAP; Kaneko et al., 1991; Fukushima et al., 1994a; Fukushima et al., 1998b; Fig. 7) revealed that *Y. pseudotuberculosis* can be separated into six genetic groups.

YPMa<sup>+</sup>/HPI<sup>+</sup>: Far East The strains belong to serotypes O:1b, 3, 5a, 5b and 15 and are isolated from patients and wild animals in the Far East

Table 9. Relationship between serotypes of *Y. pseudotuberculosis* and clinical manifestations in Japan.

Clinical manifestations	Serotypes and no. of strains													Total
	1a	1b	2a	2b	2c	3	4a	4b	5a	5b	6	7	8	
Multiple symptoms	0	8	8	6	3	1	1	57	17	26	0	0	0	127
Gastroenteritis	0	2	0	0	0	3	0	13	2	5	0	0	25	
Nongastroenteritis	0	2	1	2	1	1	0	2	1	7	0	0	17	
Kawasaki syndrome	0	0	0	2	0	0	0	6	1	3	0	0	12	
Septicemia	0	2	1	1	0	0	0	4	1	3	0	0	12	
Appendicitis	0	2	0	0	0	0	0	2	0	0	0	0	4	
Community outbreak	0	1	0	0	0	3	0	5	4	1	0	0	14	

From Tsubokura et al. (1987).

Table 10. Sources and serotypes of *Y. pseudotuberculosis*.<sup>a</sup>

Areas	Sources	Serotype																				
		Total	1a	1b	1c	2a	2b	2c	3	4a	4b	5a	5b	6	7	9	10	11	12	13	14	15
Western countries		296	58	30	0	1	33	0	161	3	0	10	0	0	0	0	0	0	0	0	0	0
Europe	Humans	20	9	5	0	0	2	0	3	0	0	1	0	0	0	0	0	0	0	0	0	0
	Domestic animals <sup>b</sup>	9	3	1	0	1	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0
	Cat	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Hares	7	2	3	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Birds	75	34	18	0	0	13	0	1	0	0	9	0	0	0	0	0	0	0	0	0	0
Australasia	Human	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Domestic animals <sup>c</sup>	131	6	1	0	0	16	0	108	0	0	0	0	0	0	0	0	0	0	0	0	0
S. America	Buffaloes	24	2	0	0	0	0	0	22	0	0	0	0	0	0	0	0	0	0	0	0	0
N. America	Humans	3	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Monkeys	22	0	0	0	0	0	0	22	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pigs	3	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Eastern countries		1939	5	213	2	12	115	65	253	104	565	208	209	50	38	10	18	12	2	4	1	53
Russia	Humans	54	0	20	0	0	0	0	11	5	18	0	0	0	0	0	0	0	0	0	0	0
	Wild mice	37	0	23	0	0	0	0	6	7	1	0	0	0	0	0	0	0	0	0	0	0
	Reindeer	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Salmon	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Environment <sup>d</sup>	14	0	3	0	0	0	0	2	3	6	0	0	0	0	0	0	0	0	0	0	0
China	Human	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pigs	3	0	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0
	Rabbits	17	0	6	0	0	2	0	4	0	0	0	3	0	0	0	0	0	0	2	0	0
	Wild mice	10	0	0	1	1	2	0	1	0	1	0	0	1	0	0	0	0	0	2	1	0
Korea	Humans	65	0	1	0	0	0	0	0	0	23	0	0	0	0	0	0	0	0	0	0	41
	Pigs	2	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
	Chicken	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	Wild mouse	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	Water	19	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	12
Japan	Humans	530	0	41	0	10	41	10	12	4	212	60	140	0	0	0	0	0	0	0	0	0
	Pigs	312	0	40	0	0	17	20	146	8	78	2	1	0	0	0	0	0	0	0	0	0
	Pork	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
	Cattle and goats	2	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Cats and dogs	69	0	18	0	0	3	3	8	1	29	4	0	2	1	0	0	0	0	0	0	0
	Zoo animals <sup>e</sup>	46	0	17	0	0	0	0	0	1	24	1	0	3	0	0	0	0	0	0	0	0
	Experimental animals <sup>f</sup>	25	0	6	0	0	1	1	3	0	2	2	8	2	0	0	0	0	0	0	0	0
	Raccoon dogs	200	0	7	0	0	25	8	8	40	60	18	14	2	2	0	15	1	0	0	0	0
	Wild animals <sup>g</sup>	55	0	1	0	0	6	1	6	2	8	9	2	13	2	3	0	2	0	0	0	0
	Wild mice	105	0	2	0	0	2	3	32	9	15	16	3	15	5	2	0	1	0	0	0	0
	Birds	10	0	1	0	0	0	0	6	0	3	0	0	0	0	0	0	0	0	0	0	0
	Water	352	0	25	1	1	15	19	3	24	73	96	38	12	28	5	2	8	2	0	0	0
	Soil	2	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Total		2235	63	243	2	13	148	65	414	107	565	218	209	50	38	10	18	12	2	4	1	53

<sup>a</sup>The strains were kindly provided by S. Aleksic, A. Borczyk, E. de Boer, D. P. Falcao, T. Honda, M. Inoue, K. Kaneko, S. Kaneko, J. Katayama, I.-K. Paik, R. Robins-Brown, Y. Ohtomo, M. Sasaki, F. N. Shubin, N. Takeda, M. Tsubokura, and R. Van Noyen.

<sup>b</sup>Horses (4), rabbits (3), pigs and guinea pigs (one each).

<sup>c</sup>Cows and domestic deer (2 each) and goats (one).

<sup>d</sup>Soil, onions, potatoes (2 each), cabbage, beets, spaghetti (one each), and swabs of table and utensils in the kitchen (2).

<sup>e</sup>Monkeys (42) and cape hyrax (5).

<sup>f</sup>Rabbits (15) and guinea pigs (10).

<sup>g</sup>Moles (24), deer (11), martens (9), boars, foxes and hares (2 each).

Fig. 4. Distribution of serotypes of *Y. pseudotuberculosis*.

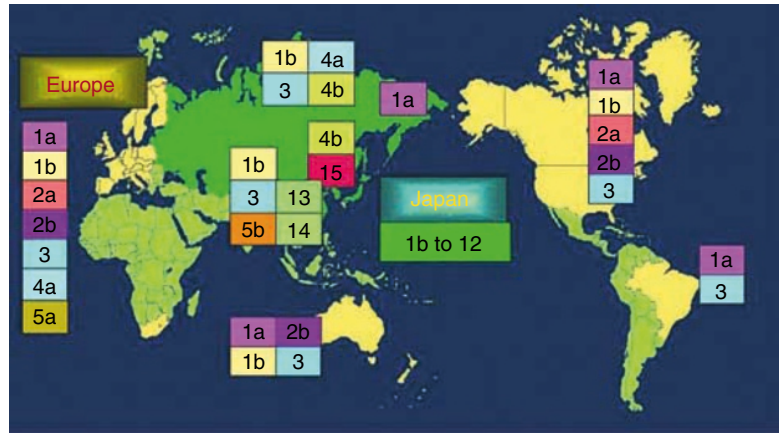


Fig. 5. Geographical distribution of *Y. pseudotuberculosis*.

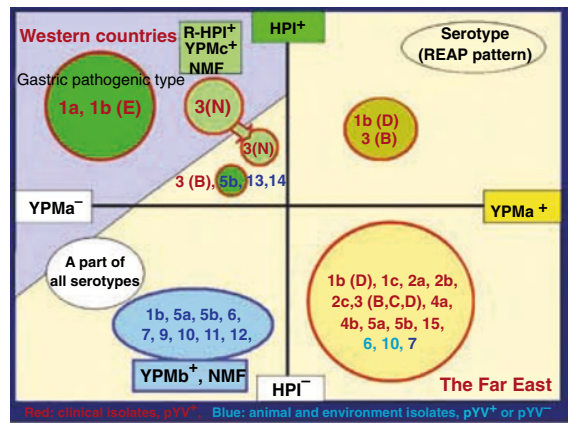
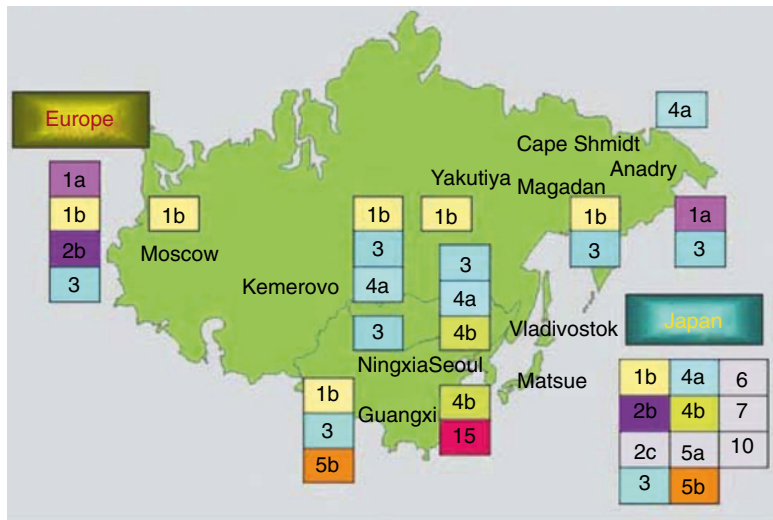


Fig. 6. Distribution of serotypes of pYV+ in *Y. pseudotuberculosis*.



(Table 11). The *virF* gene was detected in 56% of the strains.

YPM-/HPI+: European Enteropathogenic Type These strains are mostly from serotypes O:1a and 1b and are predominantly isolated from patients and wild animals in Western countries (Mair,

1965; Hubbert, 1972; Toma, 1986; Chiesa et al., 1993; Aleksic et al., 1995; Table 12). Five serotype O:1a strains were also isolated from reindeer and salmon in Far East Russia (Fukushima et al., 1998b). Eleven strains belonging to serotypes O:3, 5b, 13 and 14 from a patient and wild animals in the Far East were also in this group.

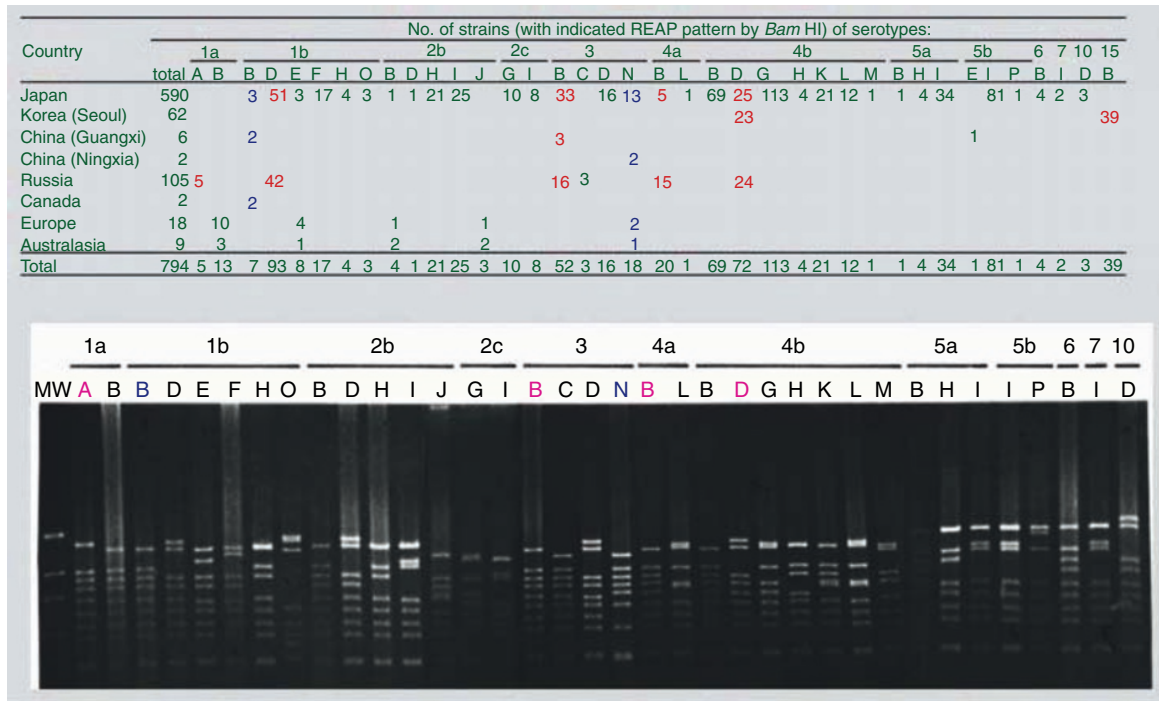


Fig. 7. Restriction endonuclease analysis of virulence plasmid DNA (REAP) patterns by *Bam* HI among serotypes isolated from various sources in eastern and western countries. From Fukushima et al. (1994a).

Table 11. Prevalence of YPMa<sup>+</sup>/HPI<sup>+</sup> *Y. pseudotuberculosis* strains.

YPMs	<i>virF</i> gene	Areas	Sources	Total	HPI <sup>+</sup> Serotype							
					1a	1b	3	5a	5b	13	14	15
YPMa <sup>+</sup>		Total		9	0	2	2	1	2	0	0	2
MF	<i>virF</i> <sup>+</sup>			5	0	0	2	1	2	0	0	0
	<i>virF</i> <sup>-</sup>			4	0	2	0	0	0	0	0	2
		China	Rabbits	2	0	1	0	0	1	0	0	0
		Korea	Humans	3	0	1	0	0	0	0	0	2
		Japan	Humans	1	0	0	1	0	0	0	0	0
			Raccoon dogs	2	0	0	0	1	1	0	0	0
			Deer	1	0	0	1	0	0	0	0	0

Abbreviations: YPMa, *Yersinia pseudotuberculosis* mitogen a; HPI, high pathogenicity island; and MF, melibiose fermenting strains.

From Fukushima et al. (2001b).

Although three strains of serotypes O:13 and 14 from wild mice in China did not have pYV and were avirulent, one strain of serotype O:3 in this group was isolated from the sputum of a patient with a fever and pneumonia in China and was virulent for mice.

The *virF* gene was detected in 49% of the strains.

REAP patterns of serotypes O:1a (Fig. 8) and 1b (Fig. 9) strains isolated in Western countries are B and E, respectively, which differ from those isolated in the Far East.

A borderline geographical distribution of organisms between both areas, Europe and the Far East, is found west of Ural Mountains (Fukushima et al., 1998b).

This group is also present in Africa, Australasia and North and South America, and might have been unknowingly introduced by human carriers or in shipments of livestock from Europe in the late 1700s and early 1800s (Slee and Skilbeck, 1992; Mollaret, 1995; Fig. 10).

**YPMa<sup>+</sup>/HPI:** Far Eastern Systemic Pathogenic Type This group (Table 13) includes many strains belonging to 16 serotypes (O:1b, 1c, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 10 and 15) from the Far East and three strains of serotype O:4a from horses in Denmark. Almost all (82%) Far Eastern strains belonged to this group. Strains of serotypes O:1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b and 15, of which serotypes O:4b and 5b are domi-

Table 12. Prevalence of YPMs<sup>-</sup>/HPI<sup>+</sup> *Y. pseudotuberculosis* strains.

YPMs	<i>virF</i> gene	Areas	Sources	HPI <sup>+</sup> Serotype									
				Total	1a	1b	3	5a	5b	13	14	15	
YPMs		Total		99	63	25	6	0	2	2	1	0	
MF	<i>virF</i> <sup>+</sup>			49	30	11	6	0	2	0	0	0	
	<i>virF</i> <sup>-</sup>			50	33	14	0	0	0	2	1	0	
		Europe	Humans	13	9	4	0	0	0	0	0	0	
			Cat	1	1	0	0	0	0	0	0	0	
			Horse	1	0	1	0	0	0	0	0	0	
			Rabbits	2	2	0	0	0	0	0	0	0	
			Guinea pig	1	1	0	0	0	0	0	0	0	
			Hares	4	2	2	0	0	0	0	0	0	
			Pigeons	6	5	1	0	0	0	0	0	0	
			Ducks	10	7	3	0	0	0	0	0	0	
			Avian	8	7	1	0	0	0	0	0	0	
			Birds	26	15	11	0	0	0	0	0	0	
			Australasia	Cows	3	2	1	0	0	0	0	0	0
				Cattle	1	1	0	0	0	0	0	0	0
				Goat	1	1	0	0	0	0	0	0	0
		Deer		2	2	0	0	0	0	0	0	0	
		S. America	Buffaloes	2	2	0	0	0	0	0	0	0	
		N. America	Humans	2	1	1	0	0	0	0	0	0	
		Russia	Reindeer	3	3	0	0	0	0	0	0	0	
			Salmon	2	2	0	0	0	0	0	0	0	
		China	Human	1	0	0	1	0	0	0	0	0	
			Rabbits	3	0	0	1	0	2	0	0	0	
			House rats	3	0	0	0	0	0	2	1	0	
		Japan	Raccoon dogs	2	0	0	2	0	0	0	0	0	
			Martens	2	0	0	2	0	0	0	0	0	

Abbreviations: YPMs, *Yersinia pseudotuberculosis* mitogens; and for other definitions, refer to footnote in Table 11.

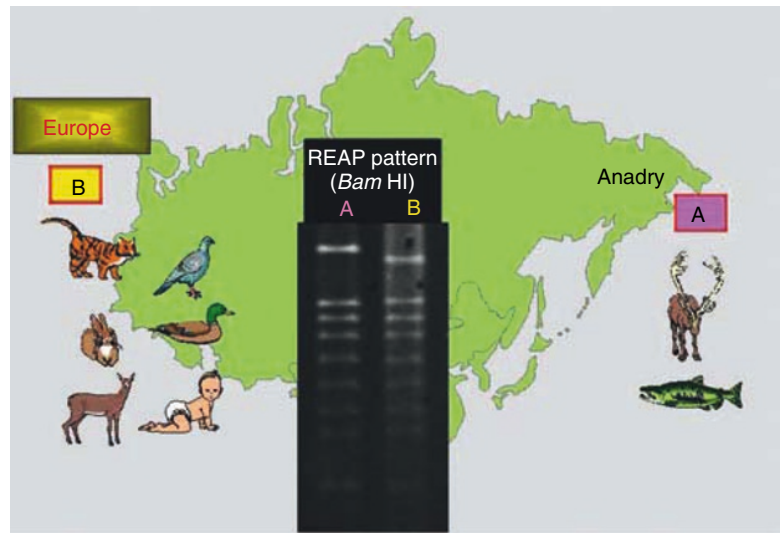


Fig. 8. Distribution of REAP (restriction endonuclease analysis of virulence plasmid DNA) patterns of *Y. pseudotuberculosis* serotype O:1a.

nant, were isolated from patients, pigs, wild animals and environments, while serotype O:6, 7 and 10 strains were isolated only from wild animals and the environment but never from clinical samples.

The *virF* gene was detected in 77% of the strains.

REAP patterns of serotype O:1b, 2b and 3 isolates in the Far East are different from

those of European isolates (Figs. 9, 11 and 12). REAP patterns of serotype O:1b, 3, 4a and 4b isolates in Korea and Russia were the same as those of Japanese isolates (Figs. 9, and 12–14). These data indicate that the origin of most *Y. pseudotuberculosis* in Japan might be the Far East region of the Eurasian continent. Spread of these organisms might have been facilitated by the connection of the

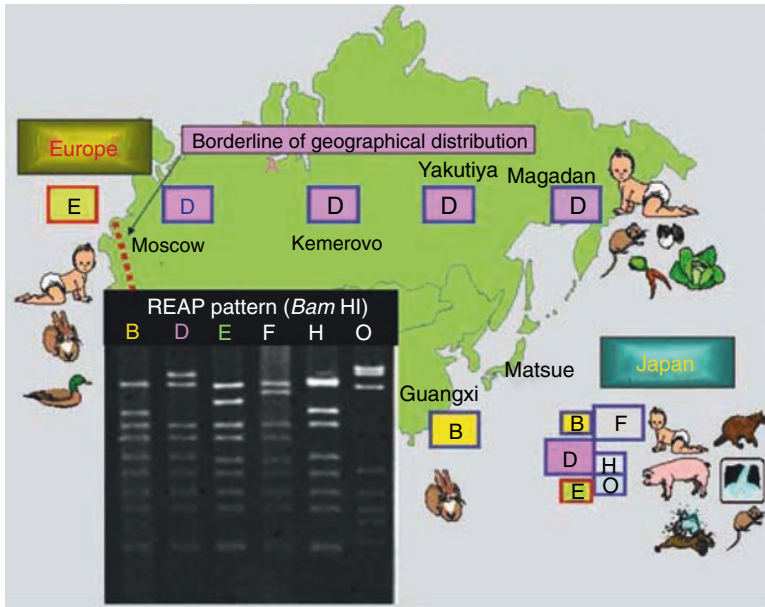


Fig. 9. Distribution of REAP (restriction endonuclease analysis of virulence plasmid DNA) patterns of *Y. pseudotuberculosis* serotype O:1b.

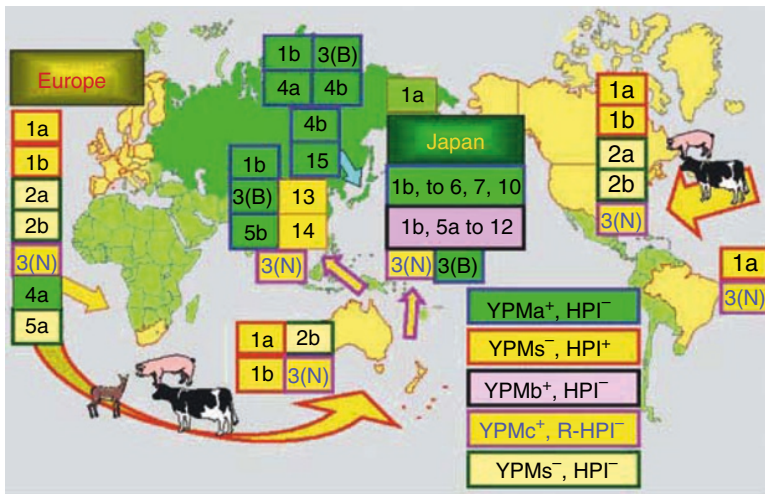


Fig. 10. Transmission of *Y. pseudotuberculosis* throughout the world.

islands of Japan to the Eurasian continent before the Ice Age.

**YPMb<sup>+</sup>/HPI<sup>-</sup>:** Nonpathogenic Japanese Strains  
 Strains belonging to the YPMb<sup>+</sup>/HPI<sup>-</sup> group are environmental isolates of serotypes O:1b, 5a, 5b, 6, 7, 9, 10, 11 and 12, and are nonpathogenic (Table 14). Those of serotype O:1b lacked pYV during stock culture and other serotypes naturally did not harbor the pYV. They were isolated from wild animals and the environment in Japan (Fukushima et al., 1990a; Nagano et al., 1997b).

**YPMC<sup>+</sup>/R-HPI<sup>+</sup>:** Low Pathogenic Strains  
 These are all serotype O:3 strains isolated from patients and animals (pigs, monkeys and livestock in Western countries, but only pigs in the Far East; Table 15). One case of human infection with a strain of this group in Japan was characterized by a terminal ileitis without systemic symptoms

(Kanazawa et al., 1974). This is in contrast with human infections due to serotype O:3 belonging to the Far Eastern systemic pathogenic group YPMa<sup>+</sup>/HPI<sup>-</sup>, which is widely distributed among pigs and wild animals in the Far East.

The pathogenicity to mice of this group is lower than that of European and Far Eastern pathogenic type (Tsubokura et al., 1984). However, this group is clearly able to produce enteritis in cattle and sheep (Slee et al., 1988; Slee and Skilbeck, 1992).

This group is present in Australasia and North and South America and might have been unknowingly introduced by human carriers or in shipments of livestock from Europe in the late 1700s and early 1800s (Slee and Skilbeck, 1992). In the Far East, these strains may have been introduced by export of pigs and pork from European pig-producing countries to Japan (Fukushima et al., 1994a; Fig. 10), same as in the

Table 13. Prevalence of YPMa<sup>+</sup>/HPI<sup>-</sup> *Y. pseudotuberculosis* strains.

YPMs	<i>virF</i> gene	Areas	Sources	HPI <sup>-</sup> Serotype																		
				Total	1b	1c	2a	2b	2c	3	4a	4b	5a	5b	6	7	9	10	11	12	13	15
YPMa+		Total		1589	201	2	11	107	63	111	96	546	163	183	13	28	0	15	0	0	0	50
Mf	<i>virF</i> <sup>+</sup>			1221	172	0	9	78	35	104	29	517	67	147	8	0	0	13	0	0	0	42
	<i>virF</i> <sup>-</sup>			368	29	2	2	29	28	7	67	29	96	36	5	28	0	2	0	0	0	8
		Europe	Horses	3	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
		Russia	Humans	54	20	0	0	0	0	11	5	18	0	0	0	0	0	0	0	0	0	0
			House rats	35	23	0	0	0	0	4	7	1	0	0	0	0	0	0	0	0	0	0
			Vegetables	13	3	0	0	0	0	1	3	6	0	0	0	0	0	0	0	0	0	0
		China	Rabbits	5	4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Wild mice	2	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
			House rat	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Korea	Humans	62	0	0	0	0	0	0	0	23	0	0	0	0	0	0	0	0	0	39
			Pig	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
			Wild mice	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
			Water	17	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	11
		Japan	Humans	517	41	0	9	41	10	8	3	212	60	133	0	0	0	0	0	0	0	0
			Pigs	214	40	0	0	17	20	61	8	68	0	0	0	0	0	0	0	0	0	0
			Cow	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Goat	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Chickens	3	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
			Cats	21	3	0	0	0	2	5	0	11	0	0	0	0	0	0	0	0	0	0
			Dogs	44	13	0	0	3	0	3	1	18	4	0	2	0	0	0	0	0	0	0
			Rabbits	15	4	0	0	1	1	0	0	2	0	7	0	0	0	0	0	0	0	0
			Pigeon	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
			Guinea pigs	5	1	0	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0
			Monkeys	40	17	0	0	0	0	0	0	19	1	0	3	0	0	0	0	0	0	0
			Cape hyraxes	5	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
			Hares	2	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
			Raccoon dogs	182	6	0	0	24	8	6	36	56	14	13	2	2	0	15	0	0	0	0
			Deer	9	0	0	0	0	0	0	0	8	1	0	0	0	0	0	0	0	0	0
			Boars	2	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
			Martens	6	0	0	0	2	0	3	1	0	0	0	0	0	0	0	0	0	0	0
			Foxes	2	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
			House rat	4	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0
			Wild mice	42	2	0	0	2	3	1	5	15	11	1	2	0	0	0	0	0	0	0
			Moles	2	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
			Wild ducks	2	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
			Water	271	20	1	1	13	18	3	21	71	69	29	1	24	0	0	0	0	0	0
			Soil	2	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0

Abbreviations: see footnote in Table 11.  
From Fukushima et al. (2001b).

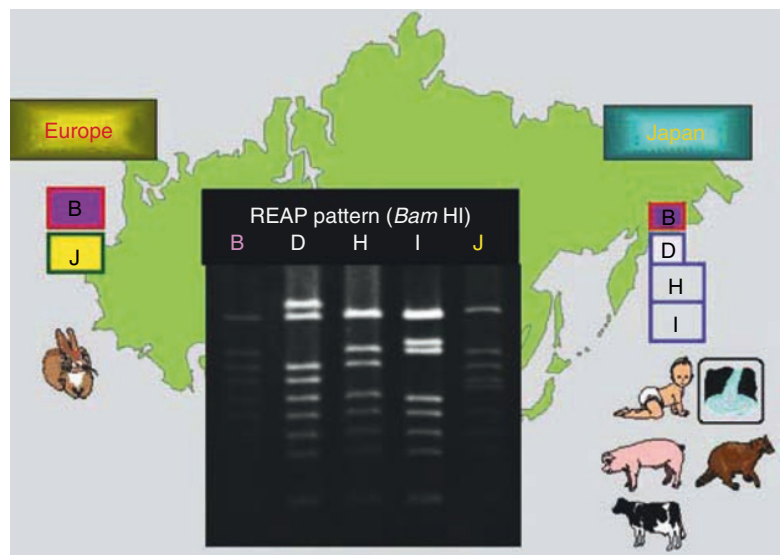


Fig. 11. Distribution of REAP (restriction endonuclease analysis of virulence plasmid DNA) patterns of *Y. pseudotuberculosis* serotype O:2b.

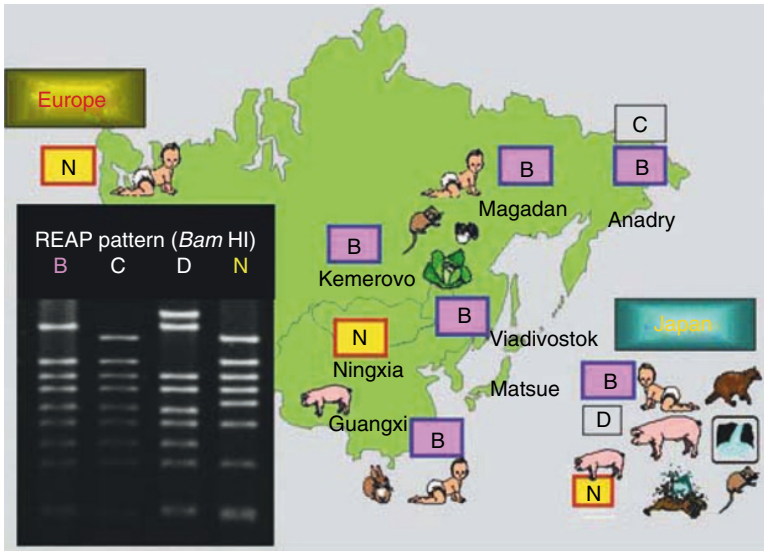


Fig. 12. Distribution of REAP (restriction endonuclease analysis of virulence plasmid DNA) patterns of *Y. pseudotuberculosis* serotype O:3.

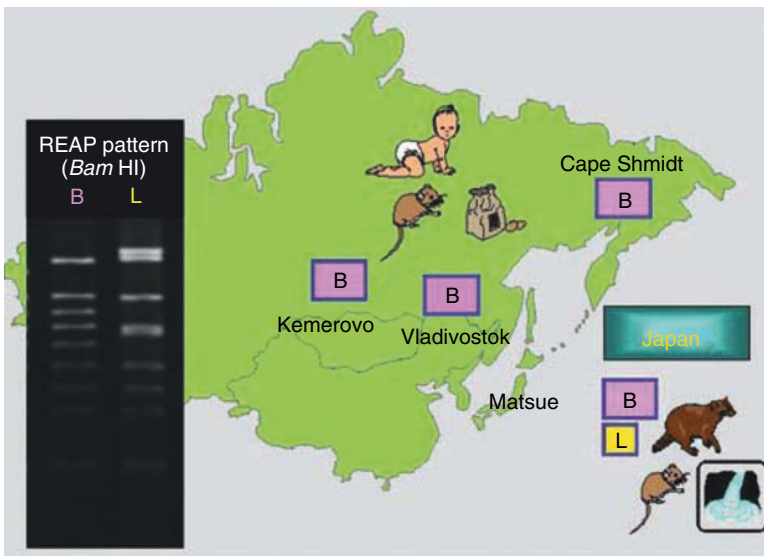


Fig. 13. Distribution of REAP (restriction endonuclease analysis of virulence plasmid DNA) patterns of *Y. pseudotuberculosis* serotype O:4a.

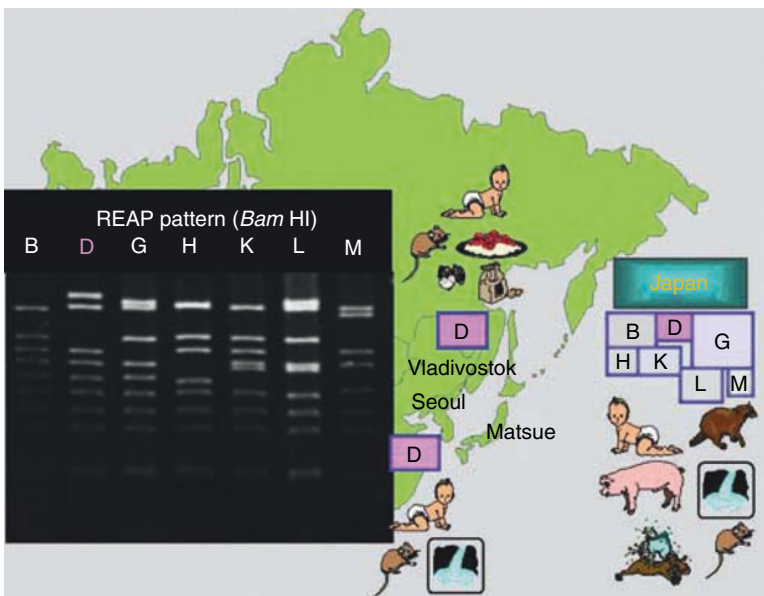


Fig. 14. Distribution of REAP (restriction endonuclease analysis of virulence plasmid DNA) patterns of *Y. pseudotuberculosis* serotype O:4b.



Table 14. Prevalence of YPMb<sup>+</sup>/HPI<sup>-</sup> *Y. pseudotuberculosis* strains.

YPMs	<i>virF</i> gene	Areas	Sources	HPI <sup>-</sup> Serotype																		
				Total	1b	1c	2a	2b	2c	3	4a	4b	5a	5b	6	7	9	10	11	12	13	15
YMPb <sup>+</sup>		Total		93	5	0	0	0	0	0	0	0	17	12	34	4	10	2	7	2	0	0
NMF	<i>virF</i> <sup>+</sup>			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	<i>virF</i> <sup>-</sup>			93	5	0	0	0	0	0	0	0	17	12	34	4	10	2	7	2	0	0
		Japan	Marten	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
			Moles	22	0	0	0	0	0	0	0	0	5	1	11	0	3	0	2	0	0	0
			Wild mice	25	0	0	0	0	0	0	0	0	3	2	12	4	3	0	1	0	0	0
			Water	45	5	0	0	0	0	0	0	0	9	8	11	0	4	2	4	2	0	0

Abbreviations: NMF, non-melibiose fermenting strains; and for other definitions, refer to footnote in Table 11.  
From Fukushima et al. (2001b).

Table 15. Prevalence of YPMc<sup>+</sup>/R-HPI<sup>+</sup> *Y. pseudotuberculosis* strains.

YPMs	<i>virF</i> gene	Areas	Sources	Total	R-HPI <sup>+</sup> Serotype O3
YPMc <sup>+</sup>		Total		235	235
NMF	<i>virF</i> <sup>+</sup>			209	209
	<i>virF</i> <sup>-</sup>			26	26
		Europe	Humans	3	3
			Pig	1	1
		Australasia	Human	1	1
			Pigs	59	59
			Cows	2	2
			Cattle	12	12
			Goats	31	31
			Deer (domestic)	4	4
		S. America	Buffalos	22	22
		N. America	Pigs	3	3
			Monkeys	22	22
		China	Pigs	2	2
		Korea	Pigs	1	1
		Japan	Human	1	1
			Pigs	71	71

Abbreviations: NMF, non-melibiose fermenting strains; and for other definitions, refer to footnote in Table 11.  
From Fukushima et al. (2001b).

case of *Y. enterocolitica* (Fukushima et al., 1997).

Strains in this group do not ferment melibiose.

The *virF* gene was detected in 89% of the strains.

REAP pattern of this group is N, which is different from that of serotype O:3 strains isolated from wild animals and from half of the pigs (Fig. 12).

**YPM<sup>-</sup>/HPI<sup>-</sup>** This group is composed of clinical strains and many environmental strains belonging to 15 serotypes (O:1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 10, 11, 13 and 15). All strains of serotypes O:2b and 5a in Western countries belong to this group (Fukushima et al., 2001b; Table 16).

The *virF* gene was detected in 50% of the strains.

Therefore, analysis of the distribution of these genetic groups suggests that *Y. pseudotuberculosis* forms two main branches that originated in the Far East and in Europe. European strains of *Y. pseudotuberculosis* belong mostly to the enteropathogenic group YPM<sup>-</sup>/HPI<sup>+</sup> (serotypes O:1a and 1b) and to the low-pathogenic type

YPMc<sup>+</sup>/R-HPI<sup>+</sup> (serotype O:3). In contrast, most Far Eastern strains belong to the YPMa<sup>+</sup>/HPI<sup>-</sup> group (serotypes O:1b, 1c, 2a, 2b, 3, 4a, 4b, 5a, 5b, 6, 10 and 15), which cause systemic infections, and to the nonpathogenic type YPMb<sup>+</sup>/HPI<sup>-</sup>.

**RESERVOIRS OF *Y. pseudotuberculosis*** *Yersinia pseudotuberculosis* is widely distributed among mammals (farm animals, pets, and wild animals) and birds in most countries with cold climates (Tables 10 and 17). The bacterium is widely spread in the environment (soil, water, vegetables, etc.) contaminated by feces of infected wild animals, and can survive in water for long periods (Dominowska and Malottke, 1971). The principal reservoirs are rodents and birds for the YPM<sup>-</sup>/HPI<sup>+</sup> and YPMa<sup>+</sup>/HPI<sup>-</sup> groups (Mair, 1973; Fig. 15), farm animals such as pigs and ruminants for the YPMc<sup>+</sup>/R-HPI<sup>+</sup> group (Slee and Skilbeck, 1992; Fig. 16), and moles for the YPMb<sup>+</sup>/HPI<sup>-</sup> group (Fig. 17).

Strains of this group do not ferment melibiose.

**YPM<sup>-</sup>/HPI<sup>+</sup> Group** Strains of serotypes O:1a and 1b in this group (Table 12 and Fig. 15) are

Table 16. Prevalence of YPM<sup>-</sup>/HPI<sup>-</sup> *Y. pseudotuberculosis* strains.

YPMs	<i>virF</i> gene	Areas	Sources	HPI <sup>-</sup> Serotype																		
				Total	1b	1c	2a	2b	2c	3	4a	4b	5a	5b	6	7	9	10	11	12	13	15
YPMs		Total		210	10	0	2	41	2	60	11	19	37	10	3	6	0	1	5	0	2	1
MF	<i>virF</i> <sup>+</sup>			106	9	0	1	18	0	48	2	17	3	7	1	0	0	0	0	0	0	0
	<i>virF</i> <sup>-</sup>			104	1	0	1	23	2	12	9	2	34	3	2	6	0	1	5	0	2	1
		Europe	Humans	4	1	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0
			Rabbit	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Hares	3	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Pigeon	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
			Avians	13	0	0	0	12	0	0	0	0	1	0	0	0	0	0	0	0	0	0
			Birds	11	2	0	0	1	0	0	0	0	8	0	0	0	0	0	0	0	0	0
		Australasia	Pigs	7	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Cows	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Goats	4	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Deer	3	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		N. America	Human	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Russia	Wild mice	2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
			Vegetables	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
		China	Pig	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
			Rabbits	7	1	0	0	1	0	3	0	0	0	0	0	0	0	0	0	0	0	2
			House rats	3	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0
			Wild mice	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Korea	Chicken	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
			Water	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
		Japan	Humans	11	0	0	1	0	0	2	1	0	0	7	0	0	0	0	0	0	0	0
			Pigs	29	0	0	0	0	0	14	0	12	2	1	0	0	0	0	0	0	0	0
			Chickens	2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
			Dogs	4	2	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
			Rabbits	2	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
			Guinea pigs	3	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
			Monkey	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
			Hares	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Raccoon dogs	14	1	0	0	1	0	0	4	4	3	0	0	0	0	1	0	0	0	0
			Deer	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
			Martens	2	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
			House rats	14	0	0	0	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0
			Wild mice	21	0	0	0	0	0	16	1	0	2	0	1	1	0	0	0	0	0	0
			Moles	2	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
			Bird	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
			Water	35	0	0	0	2	1	0	3	2	18	1	0	4	0	0	4	0	0	0

Abbreviations: MF, melibiose fermenting strains; for other definitions, refer to footnote in Table 11. From Fukushima et al., (2001b).

found in a wide variety of mammals and birds in Europe (Mair, 1965; Bercovier et al., 1978; Aldova et al., 1980; Weber and Knapp, 1981; Weber et al., 1983; Mackintosh and Henderson, 1984; Aleksic et al., 1995; Tables 18 and 19), Africa (Mollaret, 1995), Australasia (Henderson, 1983a; Henderson, 1984; Hodges et al., 1984; Slee et al., 1988; Slee and Skilbeck, 1992; Table 20), North America (Hubbert, 1972; Langford, 1972; Toma, 1986; Tables 21–23), and South America (Martins et al., 1998; Table 24). Some strains of serotype 1a were also isolated from reindeer and salmon in Far East Russia (Fukushima et al., 1998b).

Serotype O:1a and 1b strains have been isolated from farm animals (cattle, buffalo, horse, sheep, goat, domestic deer and pig), domestic pets (cat and dog), experimental animals (guinea pig, hamster and rabbit), commercially reared fur-bearers (chinchilla, mink and coypu), wild mammals (hare, rat, mouse, vole, shrew, beaver,

deer, cats, etc.), zoological animals (monkey, kangaroo, ocelot, etc.), domesticated birds (hen, turkey and duck) and wild birds (canary, pigeon, etc.; Mair, 1965; Mair, 1973; Hubbert, 1972; Langford, 1972; Hacking and Sileo, 1974). Hares and guinea pigs are most frequently affected. In France, serotype O1 strains were isolated from 25% of hares and from 2.5% of soil samples (Bercovier et al., 1978). Many cases of animal yersiniosis due to infection by this group have been recorded in Western Europe, Australasia, Brazil and the United States, e.g., cats (Mair et al., 1967; Allard, 1979; Spearman et al., 1979), cows (Mair and Harbourne, 1963; Jerrett and Slee, 1989; Welsh and Stair, 1993), goats (Karbe and Erickson, 1984), deer (Chapman et al., 1979), foxes (Black et al., 1996), hares (Wuthe et al., 1995), monkeys (Bronson et al., 1972; Chang et al., 1980; Rosenberg et al., 1980; Brack and Gatesman, 1991; Plesker and Claros, 1992), wild park animals (Welsh et al., 1992),

Table 17. Reports of isolation of *Y. pseudotuberculosis* from animals and water.

Animal source	Countries	No. of samples	No. positive	Isolation rates	Serotypes																			Serotypes (no. of strains)	Year	References					
					1	1a	1b	1c	2	2a	2b	2c	3	4	4a	4b	5	5a	5b	6	7	9	10				11	12	13	14	UT
Pigs	Japan	1796	41	2.3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1(2), 3(38), UT(1)	1972	Zen-Yoji et al., 1974		
	Japan	2041	28	1.3	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1b(5), 3(19), 4b(4)	1973-74	Tsubokura et al., 1976	
	Japan	585	12	2.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2b, 3, 4a, 4b, 5b	1984-89	Inoue et al., 1991	
	Japan	11,796	4	0.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3(4)	1978-83	Fukushima et al., 1987	
	Japan	1200	33	2.8	0	0	8	0	0	0	1	2	16	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	1b(8), 2b(1), 2c(2), 3(16), 4b(6)	1986-87	Fukushima et al., 1989
	China	2252	2	0.1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	3(1), 4b(1)	1996-97	Fukushima et al., 2001a
Italy	1874	7	0.4	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3(7)	1981-91	Chiesa et al., 1993	
Canada	544	14	2.6	0	0	0	0	0	0	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3(14)	1974-75	Toma and Deidrick, 1975	
Germany	1206	7	0.6	1	0	0	0	4	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(1), 2(4), 3(2)	1979-80	Weber and Knapp, 1981	
Czechoslovakia	318	4	1.3	3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(3), 3(1)	1974-79	Aldova et al., 1980	
The Netherlands	163	1	4.3	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3(3)	1975-76	Narucka and Westendorp, 1977	
Pigs, tonsils	Japan	1200	52	4.3	0	0	8	0	0	0	4	7	11	0	0	22	0	0	0	0	0	0	0	0	0	0	0	0	1b(8), 2b(4), 2c(7), 3(11), 4b(22)	1986-87	Fukushima et al., 1989
Italy	217	1	0.5	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2b(1)	1981-91	Chiesa et al., 1993	
Germany	480	28	5.8	15	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(15), 2(13)	1979-80	Weber and Knapp, 1981	
Germany	232	3	1.3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(3)	1982-83	Weber et al., 1983	
The Netherlands	163	7	4.3	3	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(3), 3(3), 4(1)	1975-76	Narucka and Westendorp, 1977	
Pork	Japan	125	1	0.8	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	4b(1)	1985	Fukushima, 1985	
Porcine tongue	Japan	55	1	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	4b(1)	1982-83	Shiozawa et al., 1988	
Cows	Japan	618	1	0.2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2b(1)	1977-78	Fukushima et al., 1983	
New Zealand	350	1	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	UT(1)	1984-85	Bullians, 1986	

Table 17. *Continued*

Animal source	Countries	No. of samples	No. positive	Isolation rates	Serotypes														Serotypes (no. of strains)	Year	References									
					1	1a	1b	1c	2	2a	2b	2c	3	4	4a	4b	5	5a				5b	6	7	9	10	11	12	13	14
Cattle	Australia	2639	185	7	2	0	0	0	5	0	0	0	0	177	0	0	0	0	0	0	0	0	0	0	0	0	1	1(2), 2(5), 3NMF(17), UT(1)	1985-86	Slee et al., 1988
Sheep	Australia	449	21	4.7	1	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	1(1), 3(20)	1988-89	Slee and Skilbeck, 1992	
Rabbits	Italy	70	1	1.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2b(1)	1981-91	Chiesa et al., 1993	
	New Zealand	213	4	1.9	1	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(1), 2(3)	1982-83	Mackintosh and Henderson, 1984	
Diseased	China	17	17	100	0	0	5	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	2	0	0	1b(5), 2b(2), 3(5), 5b(3), 13(2)	1990-93	Zheng et al., 1995	
Deer (domestic)	New Zealand	3810	5	0.1	2	0	0	0	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1(2), 2(1), 3(2)	1979-82	Henderson and Hemmingsen, 1983	
Diseased	New Zealand	350	65	18.6	35	0	0	0	17	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	1(35), 2(17), 3(9)	1979-82	Henderson, 1983	
		992	7	0.7	2	0	0	0	3	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1(2), 2(3), 3(2)	1984	Henderson, 1984	
Dogs	Japan	704	13	1.8	7	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	1(7), 4(8), UT(9)	1974-76	Yanagawa et al., 1978	
		1598	26	1.6	0	0	6	0	0	0	2	1	1	2	7	0	0	5	0	0	1	0	0	0	0	0	1b(6), 2b(2), 2c(1), 3(1), 4a(2), 4b(7), 5a(5), 7(1)	1980-76	Fukushima et al., 1984	
Cats	Japan	373	12	3.2	2	0	0	0	0	0	0	0	4	8	0	0	0	0	0	0	0	0	0	0	0	0	1(2), 3(4), 4(8)	1984-89	Inoue et al., 1991	
		434	4	0.9	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1b(1), 2b(1), 2c(1), 6(1)	1975-76	Yanagawa et al., 1978	
	New Zealand	18	5	27.8	3	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1(3), 2(1), 3(1)	1976-86	Fukushima et al., 1985a	
Hares	France	339	136	40.1	86	0	0	0	39	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	5	1(86), 2(39), 5(6), UT(5)	1982-83	Mackintosh and Henderson, 1984	
	Japan	221	1	0.5	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2c(1)	1976-78	Bercovier et al., 1978	
		139	2	1.4	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1b(1), 2b(1)	1981-82	Tsubokura et al., 1984	
		474	6	1.3	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1b, 2b, 2c, 4b, UT	1986-90	Fukushima et al., 1991	
	New Zealand	52	2	3.8	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(1), 2(1)	1984-89	Inoue et al., 1991	
																													1982-83	Mackintosh and Henderson, 1984



Table 17. *Continued*

Animal source	Countries	No. of samples	No. positive	Isolation rates	Serotypes														Serotypes (no. of strains)	Year	References							
					1	1a	1b	1c	2	2a	2b	2c	3	4	4a	4b	5	5a				5b	6	7	9	10	11	12
	New Zealand	90	6	6.7	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(6)	1982-83	Mackintosh and Henderson, 1984
	China	148	4	2.7	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2b(1), 3(1), 13(2)	1990-93	Zheng et al., 1995
Wild birds	France	272	3	1.1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1(2), UT(1)	1976-78	Bercovier et al., 1978
	New Zealand	302	8	2.6	4	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1(4), 2(1), UT(3)	1982-83	Mackintosh and Henderson, 1984
	Italy	900	1	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5(1)	1981-91	Chiesa et al., 1993
	Japan	259	2	0.8	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1b(1), 4b(1)	1986-90	Fukushima et al., 1990a
		108	5	4.6	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	4a, 4b	1984-89	Inoue et al., 1991
Moles	Germany	34	34		23	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(23), 2(11)	June 1980	Weber et al., 1983
	Japan	174	26	14.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1b(1), 5a(6), 5b(2), 6(13), 7(1), UT(4)	1986-89	Fukushima et al., 1990a
		528	3	0.6	0	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	3(1), 4b(2)	1987-88	Hamasaki et al., 1989
Crows	Japan	145	7	4.8	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	4b(7)	1992-93	Otsuka et al., 1994
Well water	Japan	439	10	2.3	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	2b, 2c, 4a, 4b, 5a, 5b	1984-89	Inoue et al., 1991
	Czechoslovakia	1	1		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2(1)	1976	Aldova et al., 1979
River water	Japan	3492	25	0.7	0	0	+	0	0	+	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	1b, 2a, 2b, 2c, 4a, 4b, 5a, 5b, 6	1984-89	Inoue et al., 1991
		680	175	25.7	0	0	17	0	0	0	10	7	2	0	20	45	0	84	8	15	26	0	3	7	0	1b(17), 2b(10), 2c(7), 3(2), 4a(20), 4b(45), 5a(84), 5b(8), 6(15), 7(26), 9(5), 10(3), 11(7)	1990-92	Fukushima et al., 1995
Soil	France	117	17	14.5	3	0	0	0	9	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	2	1(3), 2(9), 3(1), 4(2), UT(2)	1976-78	Bercovier et al., 1978

Symbol and abbreviation: +, UT, untyped.

Fig. 15. *Yersinia pseudotuberculosis* cycle I YPMa<sup>+</sup> and HPI<sup>-</sup> strains and YPMs<sup>-</sup> and HPI<sup>+</sup> strains.

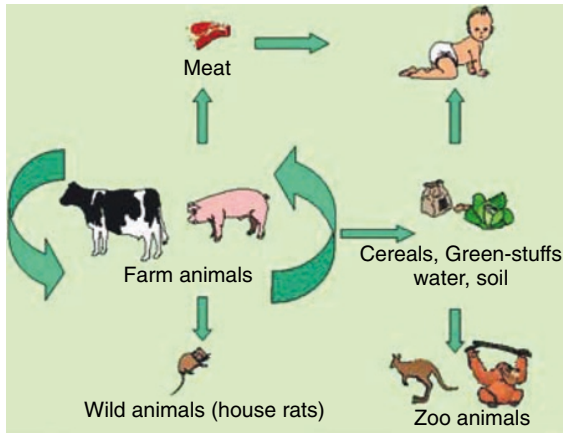
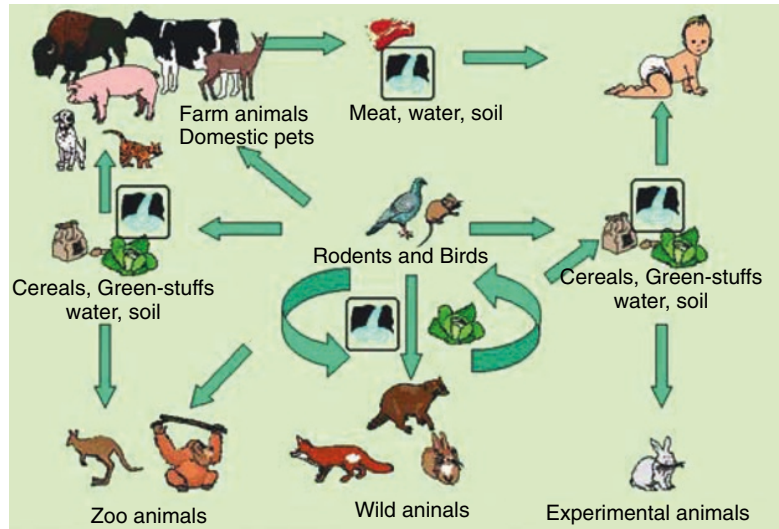


Fig. 16. *Yersinia pseudotuberculosis* cycle II YPMc<sup>+</sup> and R-HPI<sup>+</sup> strains.

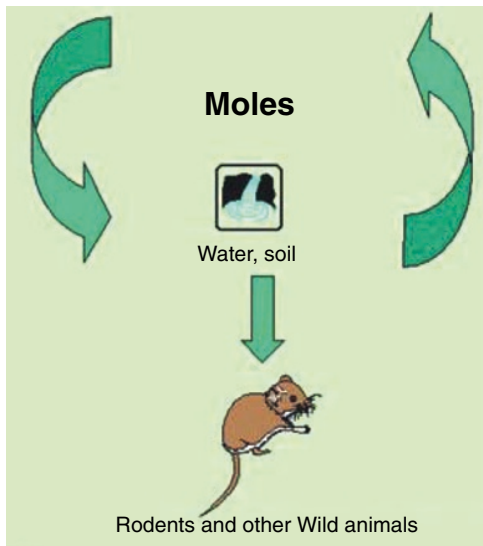


Fig. 17. *Yersinia pseudotuberculosis* cycle III YPMb<sup>+</sup> and HPI<sup>-</sup> strains.

Table 18. Distribution of serotypes of *Y. pseudotuberculosis* in Great Britain (1961–64).

Species	No. of strains in serotypes						
	1a	1b	2a	2b	3	4	5
Total	97	45	18	3	7	4	3
Mammals	58	29	12	1	7	2	3
Agouti	0	2	1	0	0	0	0
Beaver	1	0	0	0	0	0	0
Bovine fetus	2	0	0	0	0	0	0
Chinchilla	1	0	0	1	0	0	0
Coypu	5	1	2	0	0	0	0
Guinea pig	23	14	3	0	0	0	3
Hare	2	1	0	0	0	0	0
Man	5	1	1	0	0	1	0
Monkey	11	3	3	0	0	0	0
Ocelot	1	0	0	0	0	0	0
Ovine fetus	1	1	0	0	7	0	0
Rabbit	6	5	2	0	0	1	0
Vole	0	1	0	0	0	0	0
Birds	39	16	6	2	0	2	0
Barbary dove	2	0	0	0	0	0	0
Bunting	1	0	0	0	0	0	0
Canary	1	0	2	0	0	0	0
Cockatoo	1	0	1	0	0	0	0
Duck (domestic)	4	0	0	0	0	0	0
Eider	1	0	0	0	0	0	0
Grouse	0	2	0	0	0	0	0
Guinea fowl	0	2	0	0	0	0	0
Hen	5	0	0	0	0	0	0
House martin	0	0	1	0	0	0	0
Magpie	1	0	0	0	0	0	0
Oyster-catcher	1	0	0	0	0	0	0
Parakeet	2	0	0	0	0	0	0
Partridge	1	2	0	0	0	0	0
Pheasant	1	0	1	0	0	1	0
Silver bill	0	1	0	0	0	0	0
Sparrow	1	1	0	0	0	0	0
Stock-dove	1	0	0	0	0	0	0
Swallow	0	0	0	1	0	0	0
Superb tanager	1	0	0	0	0	0	0
Toucan	1	0	0	0	0	0	0
Turkey	9	8	0	0	0	0	0
Wagtail	0	0	0	0	0	1	0
Whydah	1	0	0	0	0	0	0
Wood pigeon	4	0	1	1	0	0	0
Wren	1	0	0	0	0	0	0

From Mair (1965).

Table 19. Distribution of serotypes of *Y. pseudotuberculosis* in Germany, 1983–1993.

Origin	No. of strains	Serotypes							Rough form
		1a	1b	2a	2b	3	5a	6	
Humans	54	21	10	7	3	7	2	2	2
Pig	3	1	0	0	0	2	0	0	0
Goat	2	0	0	0	0	2	0	0	0
Sheep	5	2	2	0	0	1	0	0	0
Horse	1	1	0	0	0	0	0	0	0
Dog	1	0	1	0	0	0	0	0	0
Cat	5	2	2	1	0	0	0	0	0
Duck	11	7	3	0	0	1	0	0	0
Bird	5	2	0	3	0	0	0	0	0
Parrot	3	2	1	0	0	0	0	0	0
Rabbit	2	1	0	0	0	0	0	1	0
Guinea pig	1	0	0	1	0	0	0	0	0
Monkey (zoo)	1	0	0	1	0	0	0	0	0
Wild sheep (zoo)	1	0	0	1	0	0	0	0	0
Hare	35	17	8	5	1	4	0	0	0
Deer	4	0	1	1	0	2	0	0	0
Others	4	0	1	1	0	2	0	0	0
Total	138	56	29	21	4	21	2	3	2

From Aleksic et al. (1995).

Table 20. Distribution of serotypes of *Y. pseudotuberculosis* in New Zealand.

Species	No. of strains in serotypes		
	1	2	3
Cattle	4	1	51
Sheep	1	0	7
Deer	33	9	75
Goats	2	0	11
Pigs	0	0	7
Rabbits	3	3	0
Guinea pigs	3	2	0
Birds	7	15	0

From Hodges et al. (1984).

pustard (Kormendy et al., 1988), and turkey (Wallner-Pendleton and Cooper, 1983). In New Zealand with the practice of capturing wild deer and the subsequent establishment of farmed deer, yersiniosis due to these strains emerged as a major disease (Henderson, 1983a).

Strains of serotypes O:3 and 5b in this group are found among domestic rabbits in China (Zheng et al., 1995) and wild animals such as raccoons, dogs and martens in Japan.

**YPMa<sup>+</sup>/HPI<sup>-</sup> Group** This group of strains (Table 13 and Fig. 15) is found in a wide variety of mammals and birds in the Far East: Japan (Hirai et al., 1974; Zen-Yoji et al., 1974; Tsubokura et al., 1976; Tsubokura et al., 1984; Yanagawa et al., 1978; Kaneko et al., 1979; Kaneko and Hashimoto, 1981; Fukushima et al., 1983; Fukushima et al., 1984b; Fukushima et al., 1985c; Fukushima et al., 1989b; Fukushima et al., 1990a; Hamasaki et al., 1989; Kaneuchi et al., 1989; Fukushima and

Table 21. Distribution of serotypes of *Y. pseudotuberculosis* in the United States.

Region	Species	No. of strains in serotypes					
		1a	1b	2b	3	?	
New England	Cow	0	0	0	1	0	
	Middle Atlantic	Man	1	0	0	0	0
		Pig	0	1	0	0	0
East North Central	Mammals	1	0	0	0	0	
	Bird	0	0	0	0	1	
	Kangaroo	0	1	0	0	0	
	Fallow deer	0	1	0	0	0	
West North Central	Mammals	0	0	0	0	4	
	South Atlantic	Mammals	0	1	0	0	0
		Bird	0	0	0	0	1
West South Central	Man	0	0	0	1	0	
	Mountain	Hare	1	0	0	0	0
		Squirrel	0	1	0	0	0
		Beaver	0	1	0	0	0
Pacific	Cat	1	0	0	0	0	
	Goat	0	0	0	1	0	
	Sheep	1	0	0	0	0	
	Rabbit	0	0	1	0	0	
	Chinchilla	0	0	0	1	0	
	Hare	1	0	0	0	0	
	Cottontail	0	1	0	0	0	
	Beaver	0	1	0	0	0	
	Canary	0	0	0	1	0	
	Dove	1	0	0	0	0	
	Turkey	1	0	0	0	0	
Pigeon	1	0	0	0	0		

From Hubbert (1972).

Gomyoda, 1991b; Inoue et al., 1991; Sanekata et al., 1991; Otsuka et al., 1994), China (Zheng et al., 1995), Korea (Fukushima et al., 1998b), and Far East Russia (Fukushima et al., 1998b). Three



Table 22. Distribution of serotypes of *Y. pseudotuberculosis* in Canada.

Species	No. of strains in serotypes			
	1a	1b	2a	3
Pigs	0	1	0	29
Cattle	0	3	0	15
Sheep	0	3	0	4
Goats	0	0	0	1
Felines	0	1	1	0
Deer	0	8	1	1
Hares	0	1	0	0
Rodents	0	10	0	0
Wild birds	1	3	1	1

From Toma (1986).

Table 23. Isolation of *Y. pseudotuberculosis* in Canada, 1955–68.

Species	No. of isolates
Beaver	1
Bovine	3
Chinchilla	24
Hare	1
Muskrat	1
Pig	1
Rabbit	1
Bullfinch	1
Canary	10

From Langford (1972).

Table 24. Distribution of serotypes of *Y. pseudotuberculosis* in Brazil.

Species	No. of strains in serotypes		
	1a	3 (NMF)	UT
Buffalos	3	29	2
Cattle	0	70	0
Pigs	0	1	0

Abbreviations: NMF, non-melibiose-fermenting; and UT, untyped.

From Martins et al. (1998).

strains of serotype O:4a were also isolated from horses in Denmark.

These strains are found among:

Farm animals: mainly pigs (Zen-Yoji et al., 1974; Tsubokura et al., 1976; Fukushima et al., 1989b; Fukushima et al., 1990b), but also cows (Fukushima et al., 1983), goats (Tsubokura et al., 1984), guinea pigs, and rabbits in Japan, but not in farm animals in Western countries. Serotypes O:1b, 2b, 2c, 3, 4a, 4b, 5a and 5b strains were found in 3% of slaughtered pigs in Japan. Their cecal content contained  $10^5$  cells/g (Fukushima et al., 1989b). About 75% of serotype O:3 strains



Fig. 18. Raccoon dog. This omnivorous animal belonging to a canine family is found in Japan, Korea and China. Recently the population of raccoon dogs showed a tendency to increase in mountainous areas close to residences in Japan. They eat plants and prey upon small wild animals.

isolated from pigs in Japan belong to this genetic group (Fukushima et al., 2001b)

Domestic pets: cats (0.9–3.2% in Japan), dogs (1.6–3.2% in Japan; Yanagawa et al., 1978; Fukushima et al., 1984b; Fukushima et al., 1985c), and cockatoo (Sanekata et al., 1991). Dogs and cats are infected with this organism during the cold seasons and excrete up to  $10^4$  *Y. pseudotuberculosis* cells/g of feces (Fukushima et al., 1985c)

Wild mammals: raccoon dogs (14–42.3%; Fig. 18), hares, deer, martens, foxes, rats, mice, moles, wild boars, etc. (Fukushima and Gomyoda, 1991b; Inoue et al., 1991). Serotypes O:1b, 2b, 3, 4b and 5a strains were found in 0.2–3.6% of wild mice (Kaneko and Hashimoto, 1981; Fukushima et al., 1990a) and in 3% of barn rats (Kaneko et al., 1979) in Japan. Adult and newborn wild mice are the principal reservoir of these strains and are a source of contamination for other wild animals and humans in mountainous areas (Fukushima et al., 1990a). Wild mice may excrete up to  $10^4$  *Y. pseudotuberculosis* cells/g of feces (Fig. 19). Prevalence of *Y. pseudotuberculosis* is in wild mice begins with an infection of the naïve newborn population and then quickly spreads among the naïve adult mouse population (Kaneko and Hashimoto, 1982; Fukushima, 1991a).

Zoo animals (monkey, cape hyrax, etc.).

Domestic birds (hens).

Wild birds (wild duck, crows, bunting, wagtail, pigeon, seagulls, etc.; Fukushima and Gomyoda, 1991b; Inoue et al., 1991).

*YPMc<sup>+</sup>/R-HPI<sup>+</sup>* Group This group (Table 15 and Fig. 16) is found in pigs, ruminant farm animals

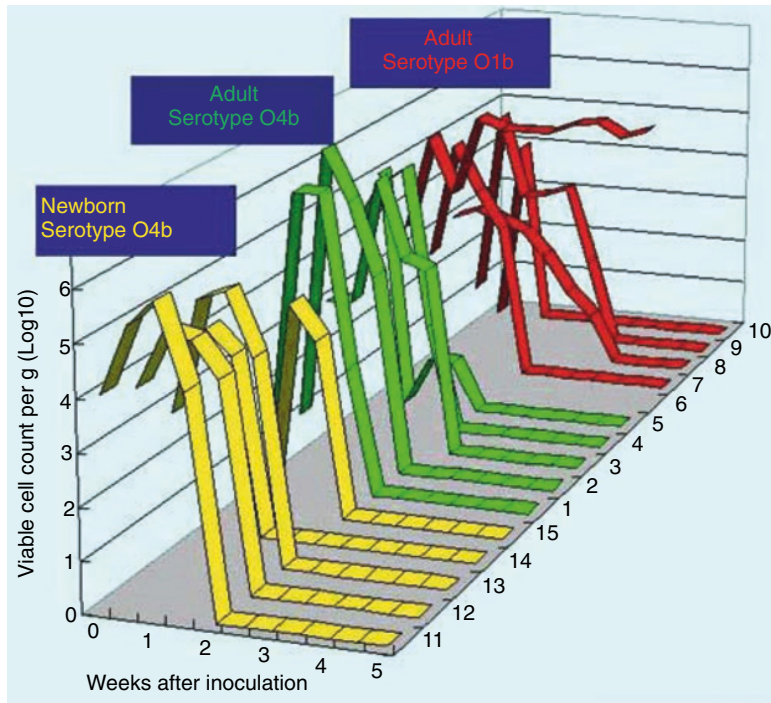


Fig. 19. Excretion of *Y. pseudotuberculosis* in the feces of *Apodemus spe-ciosus* intragastrically inoculated with  $10^9$  cells. From Fukushima (1991a).

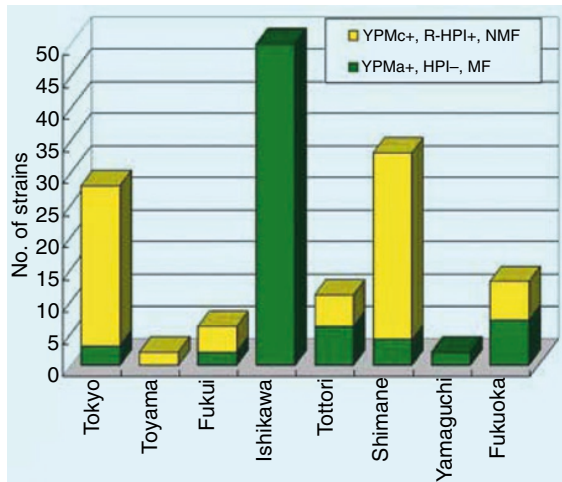


Fig. 20. Prevalence of genetic groups among *Y. pseudotuber-culosis* serotype O:3 strains isolated from pigs in Japan.

(cows, cattle, buffaloes, sheep, goats, and domestic deer), and zoo animals such as monkeys in Western countries (Tables 18–22, and 24) but only in pigs in the Far East. All serotype O:3 strains from Western countries belong to this group (Mair et al., 1979; Toma, 1986; Saridakis et al., 1988; Slee et al., 1988; Slee and Skilbeck, 1992) but half of serotype O:3 strains from Japan belong to this group (Fukushima et al., 2001b; Fig. 20).

Many cases of yersiniosis in ruminants (cattle, buffaloes, sheep and domestic deer) have been

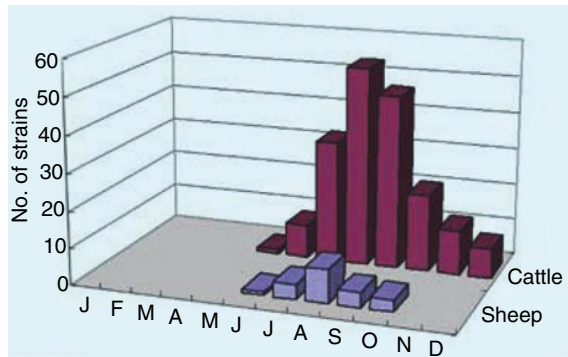


Fig. 21. Seasonal occurrence of *Y. pseudotuberculosis* serotype O:3 in sheep and cattle in Australia. From Slee et al. (Slee et al., 1988; Slee et al., 1992).

recorded in Australia (Slee et al., 1988; Slee and Skilbeck, 1992; Fig. 21), New Zealand (Henderson, 1983a; Henderson, 1984; Henderson and Hemmingsen, 1983b), and Brazil (Martins et al., 1998). This group is clearly able to produce enteritis in ruminants and is maintained in these animal flocks (Slee et al., 1988; Slee and Skilbeck, 1992). The infection in wild animals and birds is rare, but was recorded in wild mice and house rats in Czechoslovakia (Aldova et al., 1977; Urgeova and Lysy, 1995), in canaries in the United States (Hubbert, 1972), and in a wild bird in Canada (Toma, 1986). Thus, the ruminant

farm animals may be the principal reservoir of this group (Slee and Skilbeck, 1992).

**YPMb<sup>+</sup>/HPI<sup>-</sup> Group** This group (Table 14 and Fig. 17) is found in 15% of moles, which represent the reservoir and excrete 10<sup>4</sup> *Y. pseudotuberculosis* cells/g of feces (Fukushima et al., 1990a), and in river water (Fukushima, 1992; Fukushima et al., 1994b; Fukushima et al., 1995c) of the mountainous area of Japan. This group is also found in 1% of wild mice (which excrete less than 10<sup>2</sup> cells/g of feces), and was found in a marten.

**YPMa<sup>+</sup>/HPI<sup>+</sup> Group** These strains (Table 11) are found in humans, rabbits, raccoon dogs, and deer in the Far East (Fukushima et al., 2001b). The epidemiology of this group awaits confirmation from investigations of more cases of human infections.

**YPM<sup>-</sup>/HPI<sup>-</sup> Group** This group (Table 16) is found in a wide variety of mammals and birds in the Far East. The epidemiology and pathogenicity of this group also awaits confirmation.

**TRANSMISSION** Transmission of *Y. pseudotuberculosis* to humans and animals does not need a vector and occurs by contact with infected animals or by consumption of food (vegetables and meat) or water contaminated by animal reservoirs (Carniel and Mollaret, 1990; Figs. 15–17).

*Yersinia pseudotuberculosis* infection due to contaminated drinking water is frequent in mountainous areas of Japan and Korea, which have rainy weather, and where residents use unchlorinated water from wells, springs and streams.

In contrast, in Western countries, *Y. pseudotuberculosis* is rarely found in the environment, and infections occur through the consumption of greenstuffs contaminated by rodents and birds (Mair, 1965; Mair, 1973; Fukushima et al., 1998b). Moreover, contamination of pork with *Y. pseudotuberculosis* suggests that products from farm animals may also be a source of human infection (Fukushima, 1985a; Shiozawa et al., 1988).

**Birds** Wood pigeons, which are often grossly affected, have been shown to excrete huge numbers of viable organisms in their feces as they feed on green crops such as kale, cabbage and brussels sprouts (Mair, 1973), which may be heavily contaminated (Paterson and Cook, 1963). Contaminated seeds are probably also a source of infection for farm animals. In zoological gardens, contamination of food stores and water containers by rodents and birds (Mair, 1973) has been incriminated as the source of animal infections. In England, a *Y. pseudotuberculosis*

is serotype O1 strain was isolated from the feces of a man and from the excreta of his canary (Daniels, 1961).

**House Rodents** *Yersinia pseudotuberculosis* is found in house rats living in kitchens, stores, barns, warehouses, zoological gardens and slaughterhouses (Winblad, 1967; Aldova et al., 1977; Aldova et al., 1980; Kaneko et al., 1979; Mackintosh and Henderson, 1984; Fukushima et al., 1998b). Cereals, greenstuffs and water contaminated by excreta of infected rodents are a source of infections for farm animals and humans (Mair, 1973). In England, mice trapped in a birdhouse, which had suffered heavy losses from yersiniosis, were found to be carriers of *Y. pseudotuberculosis* (Mair, 1973). In Russia, the same type of *Y. pseudotuberculosis* strain was isolated from patients and from cabbages, onions, carrots, potatoes and beets stored at home, in stores or warehouses, and from peridomestic rodents captured in the same place (Fukushima et al., 1998b).

**Wild Animals** The parallel distribution of serotypes among isolates from humans, water and wild animals such as raccoon dogs and wild mice in Shimane and Okayama Prefectures in Japan demonstrates transmission to humans through water contaminated with excretion from *Y. pseudotuberculosis*-infected wild animals (Fukushima et al., 1987; Fukushima et al., 1991c; Inoue et al., 1988; Inoue et al., 1991; Sato and Komazawa, 1991; Figs. 22–25). Wild animals become infected both by preying on small wild animals infected with *Y. pseudotuberculosis* and by ingesting environmental substances contaminated with this bacterium. The question of whether the main source of infection with *Y. pseudotuberculosis* in mountainous areas is the raccoon dog or the wild mouse remains unclear (Fukushima and Gomyoda, 1991b).

In a human outbreak involving 128 patients in Okayama Prefecture, a *Y. pseudotuberculosis* strain having the same serotype as that isolated from patients was found in one third of the water samples that these patients drank. The presence of 0.2–630 *Y. pseudotuberculosis* cells/ml of untreated water was observed for at least two months in most cases. In three cases, the continuous contamination of the water with a strain of the same serotypes was observed for 12 months and then every year for four years and in another case two years later (Sato and Komazawa, 1991). In Shimane Prefecture, the identity of the distribution of REAP patterns among serotype O:1b and 4b strains isolated from humans, river water, and wild animals proves the transmission of *Y. pseudotuberculosis* by water in this mountainous area (Fukushima et al., 1991c; Fukushima and

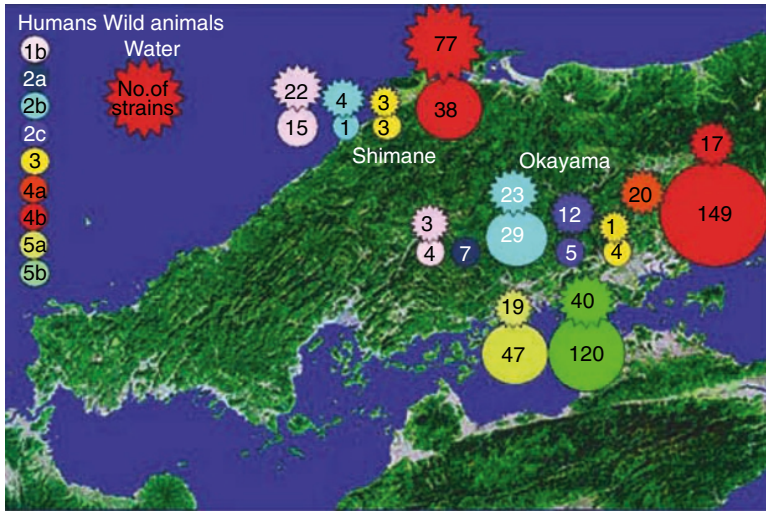


Fig. 22. Regional distribution of serotypes of *Y. pseudotuberculosis* isolated from humans, wild animals, and water between Shimane and Okayama Prefectures in Chugoku area, Japan.

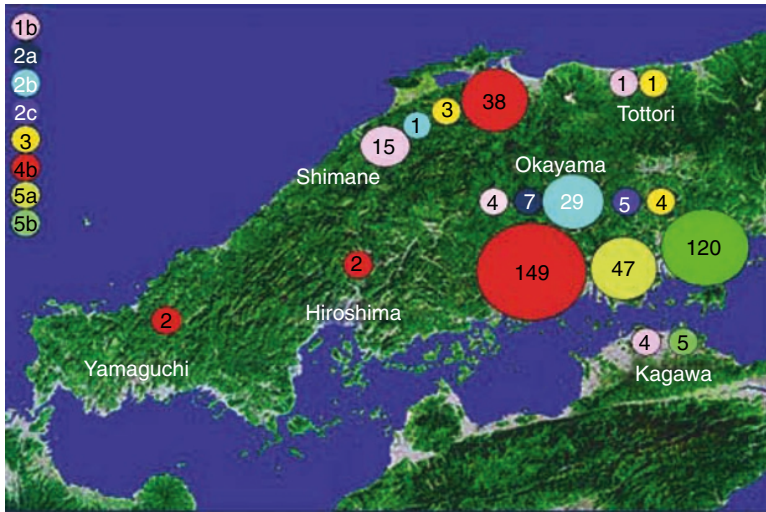
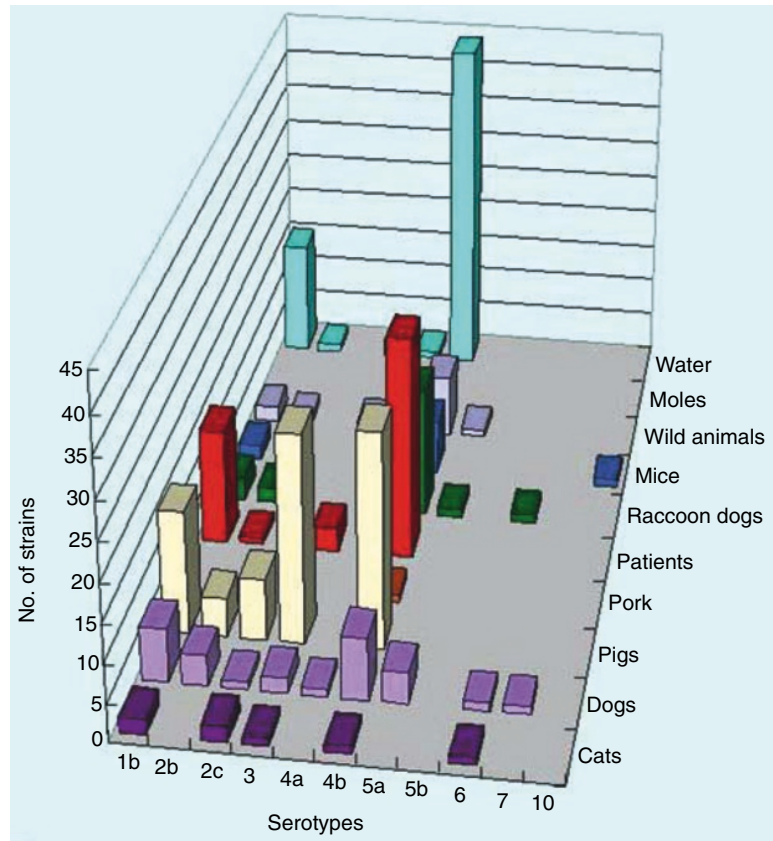


Fig. 23. Regional distribution of serotypes of *Y. pseudotuberculosis* isolated from patients in Chugoku area, Japan.



Fig. 24. Regional distribution of serotypes of YPM<sup>+</sup> *Y. pseudotuberculosis* isolated from wild animals and water in Chugoku area, Japan.

Fig. 25. Parallel distribution of serotypes of *Y. pseudotuberculosis* among isolates from patients, animals and water samples in Shimane, Japan. From Fukushima et al. (1985c).



Gomyoda, 1995b). In one valley where a 3-year-old boy had been infected with *Y. pseudotuberculosis* serotype O:4b, a strain of the same serotype was isolated from a water sample of a mountain stream from which the boy had drunk, from a wild mouse trapped in the upper part of this stream, and from excreta of a deer near the mountainous area four years later (Fukushima et al., 1988a; Fig. 26). The REAP patterns of the mice, patient, water and deer isolates were identical. These data suggest the potential for transmission of *Y. pseudotuberculosis* to humans through water contaminated by wild animals carrying this species.

**Domestic Animals** Predators such as cats are infected directly by rodents and birds, and may thus constitute an important link between the natural reservoirs and man (Mair, 1973). The apparently healthy dogs infected with *Y. pseudotuberculosis* excrete  $10^5$  cells/g of feces (Fukushima et al., 1984b) and cats and dogs with diarrhea excrete  $10^{7-8}$  cells/g of feces (Fukushima et al., 1989a). A laborer died of *Y. pseudotuberculosis* septicemia eight days after working in a garden where the soil had been fouled by excreta of an infected cat with diarrhea (Paul and Weltman, 1934). In Japan, a 1-year-old boy infected

with *Y. pseudotuberculosis* serotypes O:1b and 3, and his 3-year-old brother infected with *Y. pseudotuberculosis* serotype O:1b drank water from puddles in a garden in their housing area (Fukushima et al., 1989a). Strains with REAP patterns identical to those of the patients were isolated from the soil of the dried puddles, and from feces and the sandbox of a stray cat. This case provided evidence for the transmission of *Y. pseudotuberculosis* through water, sand, and soil contaminated by cat feces (Fig. 27).

**Farm Animals** The preponderance of serotype O:3 *Y. pseudotuberculosis* belonging to YPMc<sup>+</sup>/R-HPI<sup>+</sup> group in ruminant animals and pigs was noted in Australia (Slee et al., 1988; Slee and Skilbeck, 1992; Fig. 21), New Zealand (Henderson, 1983a; Henderson, 1984; Henderson and Hemmingsen, 1983b; Table 20), Brazil (Martins et al., 1998; Table 24) and Canada (Toma, 1986; Table 22), and prevalence of this serotype in some Japanese pigs was noted (Tsubokura et al., 1984; Nagano et al., 1997a; Fukushima et al., 2001b; Table 25). In contrast, this serotype is uncommon in birds, rodents and other animals in which YPM<sup>-</sup>/HPI<sup>+</sup> group in Western countries and YPMa<sup>+</sup>/HPI<sup>-</sup> group in the Far East are usually predominant. This serotype O:3 was also iso-

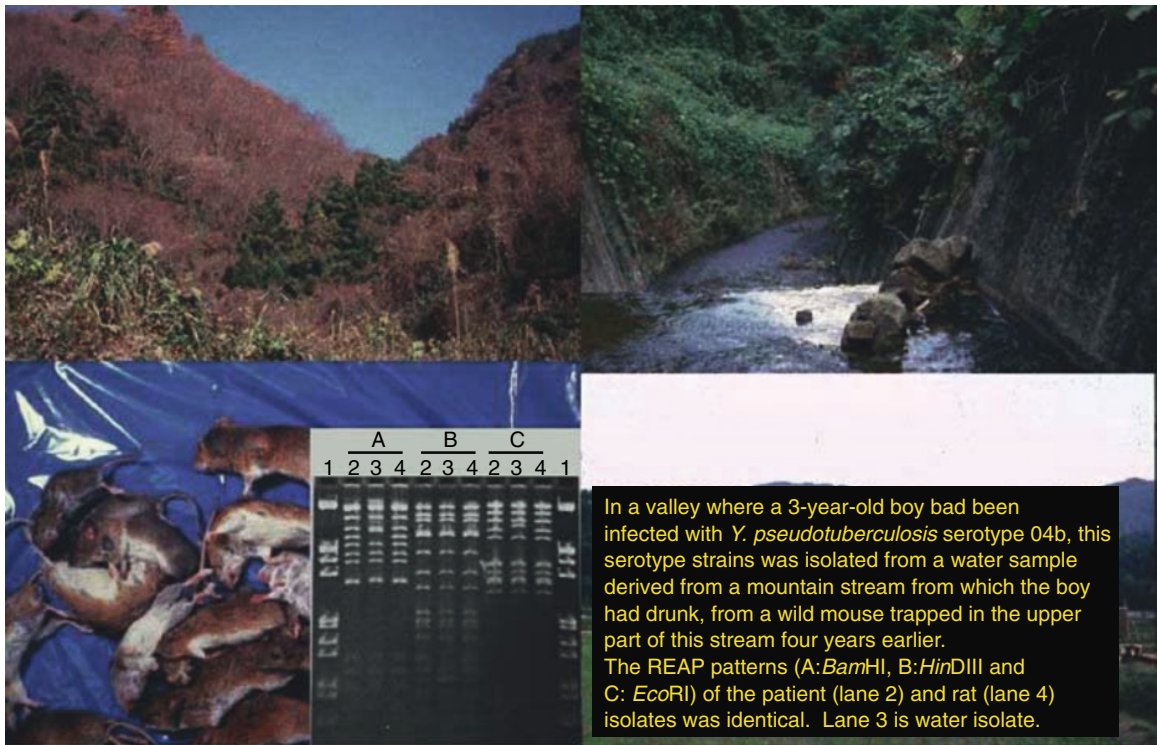


Fig. 26. *Y. pseudotuberculosis* infection contracted through water contaminated by wild animals. From Fukushima et al. (1988a).

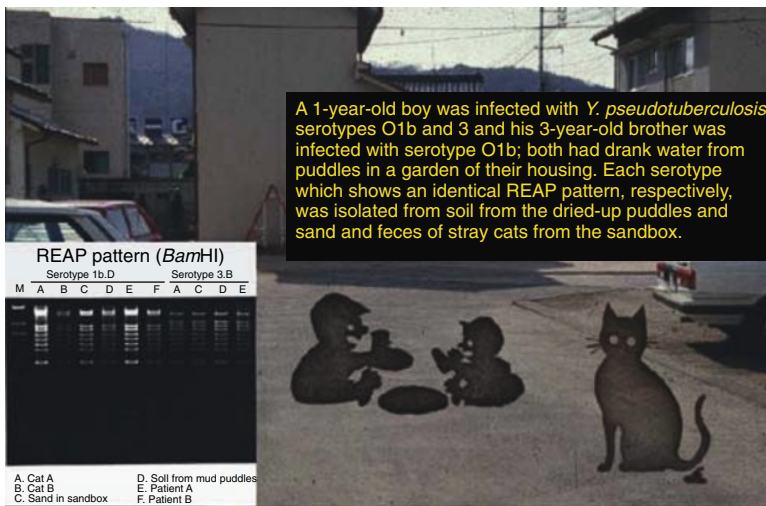


Fig. 27. Cat-contaminated environmental substances lead *Y. pseudotuberculosis* infection in children. From Fukushima et al. (1989a).

lated from humans (Kanazawa et al., 1974; Toma, 1986; Aleksic et al., 1995), suggesting that ruminant animals (Slee et al., 1988), pigs (Tsubokura et al., 1984), or their products may be the source of human infections.

The general environment of farms is naturally contaminated with excreta of wild animals such as rodents (Kaneko et al., 1979; Kaneko and Hashimoto, 1982) and birds (Mair, 1973), which carry strains of the YPM<sup>-</sup>/HPI<sup>+</sup> and YPM<sup>+</sup>/HPI<sup>-</sup>

groups in Western countries and the Far East, respectively. In Australia, serotype O:1 is occasionally isolated from sheep, suggesting that sheep are not a permanent reservoir for this bacterium (Slee and Skilbeck, 1992). In Japan, strains of the YPM<sup>+</sup>/HPI<sup>-</sup> group have been isolated from 4.5 to 6% of slaughtered pigs from 13% of pig farms (Fukushima et al., 1989b; Fig. 28). Retail pork meat was also found contaminated with strains of serotype O4b (Fukushima,

Table 25. Reports of isolation of *Y. pseudotuberculosis* from animals and water in Japan and China.

Countries	Animals	No. of samples	No. positive	Isolation rates	Serotypes														References											
					1	1a	1b	1c	2	2a	2b	2c	3	4	4a	4b	5	5a		5b	6	7	9	10	11	12	13	14	UT	
Japan	Pigs	1796	41	2.3	2	0	0	0	0	0	38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1972	Zen-Yoji et al., 1974	
		2041	28	1.3	0	0	5	0	0	0	0	0	19	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	1973-74	Tsubokura et al., 1976
			585	12	2.1	0	0	0	0	0	0	+	0	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991
			11796	4	0.03	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1978-83	Fukushima et al., 1985b
			1200	33	2.8	0	0	8	0	0	0	1	2	16	0	0	6	0	0	0	0	0	0	0	0	0	0	0	1986-87	Fukushima et al., 1989
		Pigs,	1200	52	4.3	0	0	8	0	0	0	4	7	11	0	0	22	0	0	0	0	0	0	0	0	0	0	0	1986-87	Fukushima et al., 1989
		Tonsils	125	1	0.8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1985	Fukushima 1985
		Pork	55	1	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1982-83	Shiozawa et al., 1988
		Porcine tongue	618	1	0.2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1977-78	Fukushima et al., 1983
		Cows	704	13	1.8	7	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	9	1974-76	Yanagawa et al., 1978
	Dogs	1598	26	1.6	0	0	6	0	0	0	2	1	1	2	7	0	0	5	0	0	1	0	0	0	0	0	0	1980-86	Fukushima et al., 1984	
		176	5	2.8	0	0	+	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991	
	Cats	373	12	3.2	2	0	0	0	0	0	0	0	4	8	0	0	0	0	0	0	0	0	0	0	0	0	0	1975-76	Yanagawa et al., 1978	
		434	4	0.9	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1976-86	Fukushima et al., 1985a	
	Hares	221	1	0.5	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1981-82	Tsubokura et al., 1984	
		139	2	1.4	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1986-90	Fukushima et al., 1991	
	Marten	474	6	1.3	0	0	+	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	+	1984-89	Inoue et al., 1991	
		34	1	2.9	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1986-90	Fukushima et al., 1991	
	Deer	19	5	26.3	0	0	0	0	0	0	+	+	+	0	+	0	0	0	0	0	0	0	0	0	0	0	+	1984-89	Inoue et al., 1991	
		215	8	3.7	0	0	0	0	0	0	0	0	1	0	0	6	0	1	0	0	0	0	0	0	0	0	0	1986-90	Fukushima et al., 1991	
	Wild boar	114	6	5.3	0	0	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991	

Table 25. *Continued*

Countries	Animals	No. of samples	No. positive	Isolation rates	Serotypes														References												
					1	1a	1b	1c	2	2a	2b	2c	3	4	4a	4b	5	5a		5b	6	7	9	10	11	12	13	14	UT		
	Monkey	23	2	8.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991
	Fox	39	3	11.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991
	Raccoon dogs	164	23	14	0	0	3	0	0	0	2	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1986-90	Fukushima et al., 1991
	Wild mice	390	165	42.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991
		495	1	0.2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1978	Kaneko and Hashimoto, 1981
		1530	44	2.9	0	0	2	0	0	0	1	3	0	0	3	7	0	13	1	12	0	0	0	0	0	0	0	0	4	1986-89	Fukushima et al., 1990a
		669	25	3.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991
		223	1	0.4	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	15	0	0	0	0	0	0	0	0	1989-90	Inuma et al., 1992
	Moles	174	26	14.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1986-89	Fukushima et al., 1990a
	House rats	270	8	3	0	0	1	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1976-77	Kaneko et al., 1979
	Wild birds	259	2	0.8	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1986-90	Fukushima et al., 1990a
		108	5	4.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991
		528	3	0.6	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1987-88	Hamasaki et al., 1989
	Crows	145	7	4.8	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	1992-93	Otsuka et al., 1994
	Well water	439	10	2.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991
	River water	3492	25	0.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1984-89	Inoue et al., 1991
		680	175	25.7	0	0	17	0	0	0	10	7	2	0	20	45	0	84	8	15	26	0	3	7	0	0	0	0	0	1990-92	Fukushima et al., 1995
China	Pigs	2252	2	0.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1996-97	Fukushima et al., 2001a
	Rabbits	17	17	100	0	0	5	0	0	0	2	0	5	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	1990-93	Zheng et al., 1995
		148	4	2.7	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1990-93	Zheng et al., 1995

Symbol and abbreviation: +, ••, and UT, untyped.



1985a; Shiozawa et al., 1988). When infected pigs were kept in a slaughterhouse lairage overnight, the oral cavity and skin of pigs from uninfected farms were contaminated with *Y. pseudotuberculosis* by contact and ingestion of bacteria from the floor of the lairage contaminated by the excreta of the infected pigs that harbored 10<sup>5</sup> cells/g of cecal contents (Tables 26 and 27). This contamination, occurring at the slaughterhouse, is of prime importance and should be taken into account to control the contamination of carcasses by *Y. pseudotuberculosis* (Fukushima et al., 1989b). This contamination extended to retail meat and the pork products (Fukushima et al., 1990b). *Yersinia pseudotuberculosis* serotype O:4b was isolated from raw pork (ground pork and tongue) from retail shops in Japan (Fukushima, 1985a; Shiozawa et al., 1988).

**SEASONAL DISTRIBUTION** The incidence of animal (Mair, 1965; Slee et al., 1988; Fukushima et al., 1990a; Slee and Skilbeck, 1992) and human diseases (Mair, 1965; Sato, 1987a; Fukushima and Gomyoda, 1991b; Aleksic et al., 1995) reach

a peak at the same time during late autumn and spring with cool and damp weather (Figs. 3, 21, 29, and 30). *Yersinia pseudotuberculosis* infection in wild mice begins with a contamination of the naïve newborn population in late autumn and then spreads among the *Y. pseudotuberculosis* naïve newborn and adult mice population during winter, spring and early summer (Kaneko and Hashimoto, 1982; Fukushima and Gomyoda, 1991b; Fig. 31). The seasonal incidence of this organism in farm animals, such as cattle, does not reflect the availability of susceptible host animals. There is, however, no evidence in the literature to indicate how *Y. pseudotuberculosis* survives over warm seasons when infection appears to be rare (Slee et al., 1988).

**DIFFERENTIAL GEOGRAPHICAL DISTRIBUTION DUE TO MUTUAL EXCLUSION OF THE PATHOGENIC YERSINIA** The plague bacillus, *Yersinia pestis*, has been one of the most devastating infectious agents in human history. Plague is essentially a disease of wild rodents that is transmitted to human and other animals by fleas (Carniel and Mollaret, 1990; Guiyoule et al., 1994). The closely related species *Y. enterocolitica* and *Y. pseudotuberculosis* are also pathogens of humans and cause zoonotic infections with the major reservoirs being rodents, domestic animals, and birds. Results of DNA hybridization studies have shown a close relation between *Y. pestis* and *Y. pseudotuberculosis* (Bercovier et al., 1980b). The immunity to plague after immunization with live *Y. pseudotuberculosis* is retained for a long time (Wake et al., 1978). This protection against *Y. pestis* is found in mice after oral administration of *Y. enterocolitica* serotypes O:3, O:5, 27 and O:9 (Alonso et al., 1980; Mazigh et al., 1984).

The enteropathogenic yersiniae, which are widespread among mammalian populations in various parts of the world, might serve as a silent but efficient barrier against *Y. pestis* spread from natural foci (Alonso et al., 1980). *Yersinia pseudotuberculosis* and *Y. enterocolitica* sero-

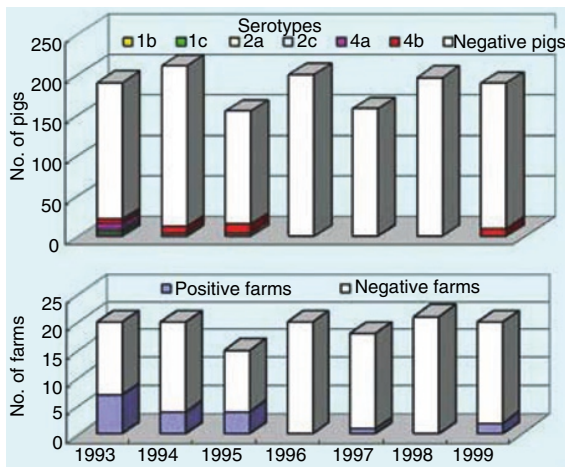


Fig. 28. Prevalence of *Y. pseudotuberculosis* in slaughtered pigs in Shimane, Japan.

Table 26. Contamination with *Y. pseudotuberculosis* of 550 pigs kept in slaughterhouse lairage.

Isolation from two samples of pigs				Serotypes					
Cecal contents	Oral cavity	Skin	No. of cases (%)	1b	2b	2c	3 MF <sup>a</sup>	3 NMF <sup>a</sup>	4b
+	-	-	19 (3.5)	7	1	0	0	9	2
+	+	-	2 (0.4)	1	0	0	0	0	1
-	+	-	28 (5.1)	7	2	2	3	0	14
-	+	+	1 (0.2)	0	0	0	1	0	0
-	-	+	8 (1.5)	1	0	0	0	7	0

Abbreviations: Refer to footnote in Table 11.

<sup>a</sup>R-HPI<sup>+</sup>, YPMc<sup>+</sup>, non-melibiose-fermenting strains.

From Fukushima et al. (1989).

Table 27. Isolation of *Y. pseudotuberculosis* from cecal contents of pigs from infected farms and of pigs supplied from uninfected farms and contaminated in slaughterhouse lairage.

Farms	Infected farms												Uninfected farms																		
	<i>Y. enterocolitica</i>						<i>Y. pseudotuberculosis</i>						<i>Y. pseudotuberculosis</i>																		
	No. of pigs examined	No. of positive pigs	Biotype			No. of positive pigs	%	Serotypes			No. of positive pigs	%	Viable cell counts per g(log <sub>10</sub> )			No. of positive pigs	Serotypes														
			4	3	S-3			1b	3	NMF <sup>a</sup>			4b	<2	2		3	4	5	6	1b	2b	2c	3	NMF	4b					
A0	78	15	19.2	15	0	0	0	0	2	2.6	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0			
B	36	2	5.6	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
C	29	4	13.8	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.9	2	0	0	0	0	0	0	0		
D	28	5	17.9	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
E	8	2	25.0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
F	5	2	40.0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
G	1	1	100	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
H	1	1	100	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
I	119	9	7.6	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
J	63	2	3.2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
K	41	2	4.9	0	2	0	0	0	3	4.8	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	
L	12	4	33.3	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
M	5	1	20.0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	3	0	0	0	0	
N	3	3	100	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	1	0	0	0	0	
O	9	5	55.6	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
P	46	0	0	0	0	0	0	0	3	6.5	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Q	32	0	0	0	0	0	0	0	2	6.3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R	38	0	0	0	0	0	0	0	6	15.8	0	6	0	0	5	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
S	39	0	0	0	0	0	0	0	3	7.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
U	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.0	1	0	0	0	0	0	0	0	0	0
V	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.0	0	1	0	0	0	0	0	0	0	0
W	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10.5	0	0	2	0	0	0	0	0	0	0
X	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10.0	0	0	0	1	0	0	0	0	0	0
Y	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.3	0	0	0	0	0	0	0	0	0	0
Other 71 farms	471	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.3	0	0	0	0	0	0	0	0	0	0
Total	1200	77	6.4	32	21	5	19	1.6	5	11	3	0	9	1	4	0	3	2	2	14	1.2	3	1	2	5	3	0	0	0	0	0

Abbreviations: See footnote in Table 12.  
<sup>a</sup>R-HPI<sup>+</sup>, YPMc<sup>+</sup>, non-melibiose-fermenting strains.  
From Fukushima et al. (1990).

types O3 and O9 are widely distributed throughout Europe and Asia and may thus protect wild rodents against *Y. pestis* infection (Mollaret, 1995). The third plague pandemic began in the Yunnan region of China and reached Hong Kong in 1894. The existence of steamships and railways greatly favored a rapid spread of plague on the five continents and the colonization of new geographic areas (e.g., the United States, South America, South Africa, and Madagascar; Guiyoule et al., 1994; Mollaret, 1995). The arrival of plague to European harbors was not followed by penetration into or establishment on the continent, possibly because rodents in Europe had become immunized by *Y. pseudotuberculosis* and subsequently resistant to plague (Mollaret, 1995). The same phenomenon was also observed in Japan. The first plague victim was identified in a steamship coming from Hong Kong to Nagasaki in 1890. Later, the disease reached harbors in Tokyo, Yokohama, Osaka, and Kobe and was prevalent among 2905 persons and 24,528

rats in Japanese harbors from 1899 to 1926 (Morita, 1995), but the plague did not penetrate into or become established in Japan, where *Y. pseudotuberculosis* might have been prevalent among wild animals from the Ice Age (Fukushima et al., 1998b). Human infection with *Y. pseudotuberculosis* in Japan was first reported in 1909 (Saisawa, 1913).

*Yersinia pseudotuberculosis* serotypes O:1a and 1b of the YPM<sup>-</sup>/HPI<sup>+</sup> group and serotype O:3 of the YPMc<sup>+</sup>/R-HPI<sup>+</sup> group spread to new continents, where enteropathogenic yersiniae are rare, following European emigrations since the eighteenth century (Slee and Skilbeck, 1992; Mollaret, 1995). Serotype O:3 strains (YPMc<sup>+</sup>/R-HPI<sup>+</sup> group) were transported to the Far East by the import of pigs and their products from Western pig-producing countries since 1970s, and the same is true for *Y. enterocolitica* (Fukushima et al., 1997; Fig. 10).

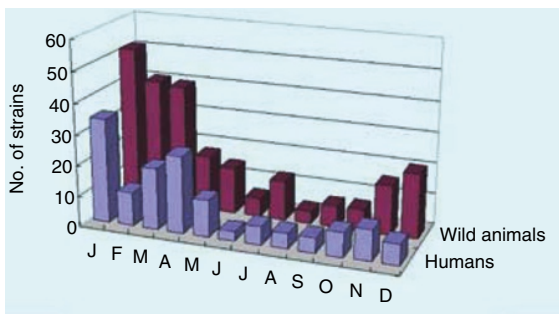


Fig. 29. Monthly distribution of *Y. pseudotuberculosis* in humans and other animals in Britain 1961–1971. From Mair (1973).

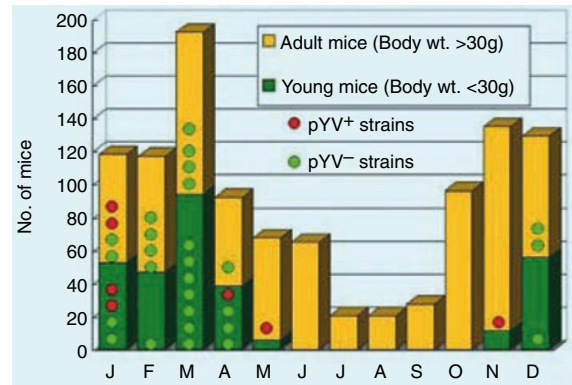


Fig. 31. Relationship between breeding season and the incidence of *Y. pseudotuberculosis* in *A. speciosus*. From Fukushima et al. (1990a).

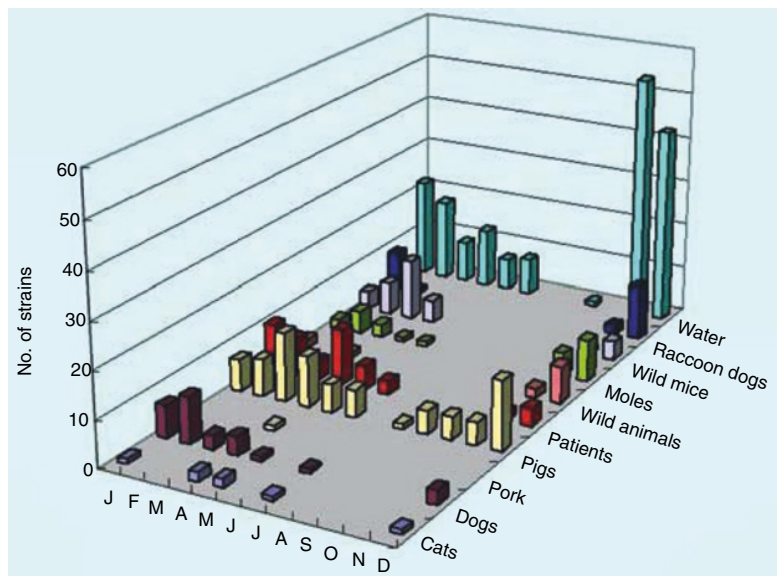


Fig. 30. Monthly distribution of *Y. pseudotuberculosis* in patients, animals and water in Shimane, Japan. From Fukushima et al. (1985c).

An effective and solid barrier against the spread of certain species or serotypes of *Yersinia* by some predominant types of *Yersinia* among domestic and wild animals is suggested by the following observations.

*In China* In Haiyuan county of the Ganning loess plateau plague focus, central China, where the main reservoir of *Y. pestis* is Mongolian ground squirrel living in farmlands and prairies, *Y. enterocolitica* O:9 is prevalent among pigs, dogs, hares and small rodents in and around a residential area but rarely among Mongolian ground squirrels (Fukushima et al., 2001a). The prevalence of *Y. pseudotuberculosis* is, however, rare among animals in this area. In Southern China, Guangxi and Shanghai, *Y. pseudotuberculosis* serotype O:1b, 1c, 2a, 2b, 3, 4b, 5b and 6 strains are prevalent among rabbits, house rats, and wild rats (Zheng et al., 1995) and *Y. enterocolitica* biotype 3/serotype O:3 strains are prevalent among pigs, rabbits, and house rats (Zheng, 1987; Zheng and Xie, 1996). These findings suggest the mosaic distribution of *Y. pestis* and two closely related species, *Y. pseudotuberculosis* and *Y. enterocolitica*, among wild and domestic animals in mainland China.

*In Brazil* *Yersinia pseudotuberculosis* is frequently distributed among domestic animals such as cattle, buffalos and pigs in the *Y. pestis* free areas, but never distributed in natural plague foci (D. P. Falcao, personal communication).

*In Japan* In western Japan where many cases of human *Y. pseudotuberculosis* infections have been reported, *Y. pseudotuberculosis* is frequently found in wild and domestic animals and in natural water, but pathogenic *Y. enterocolitica* are never isolated. Furthermore, although the most predominant serotype of clinical and animal isolates in Okayama (Sato, 1987b) and Shimane Prefectures (Fukushima et al., 1985b) was serotype O4b, the next four predominant serotypes differed between both areas (Fig. 22). Serotype O:2b, 5a and 5b strains are frequently found in humans and wild animals in Okayama (Inoue et al., 1991) but are rare in Shimane. In contrast, serotype O:1b strains are frequently found in humans and wild animals in Shimane (Fukushima and Gomyoda, 1991b) but rare in Okayama. This phenomenon was strengthened by the analysis of the REAP types of *Y. pseudotuberculosis* in a limited area (Fukushima et al., 1991c; Fukushima and Gomyoda, 1995b; Fig. 32). In Shimane Prefecture, almost all isolates from

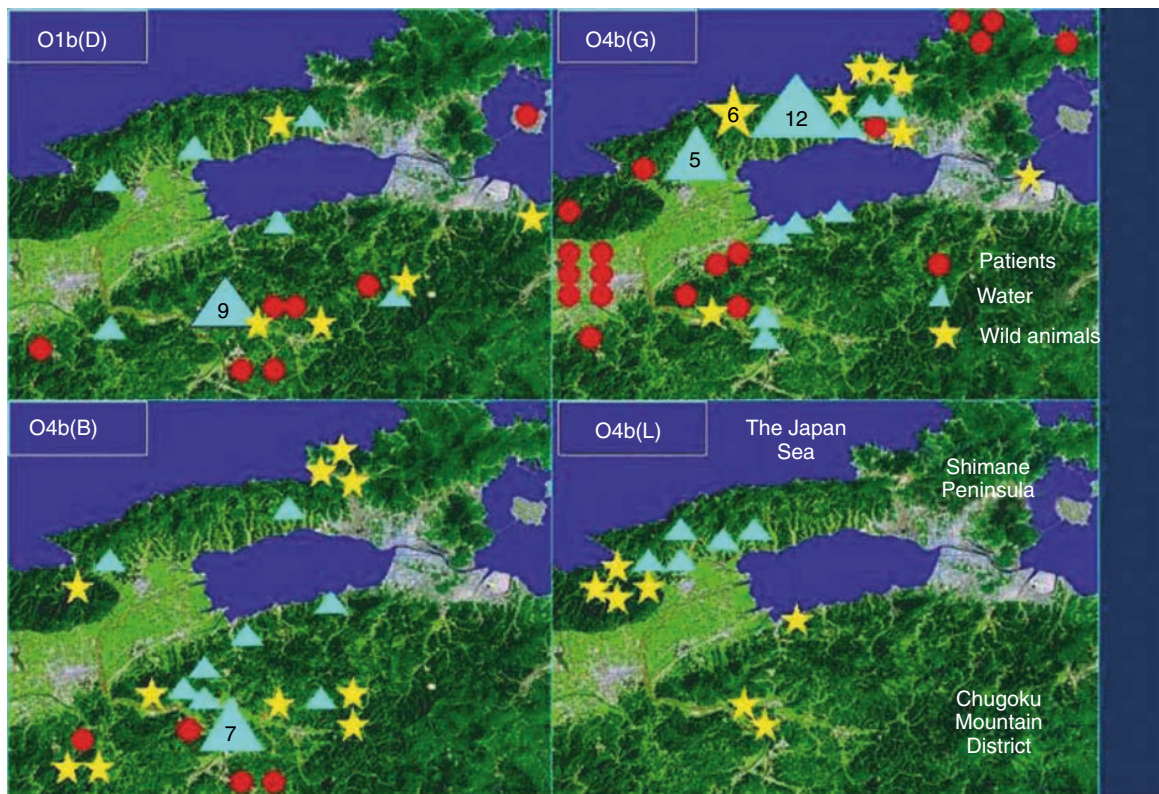
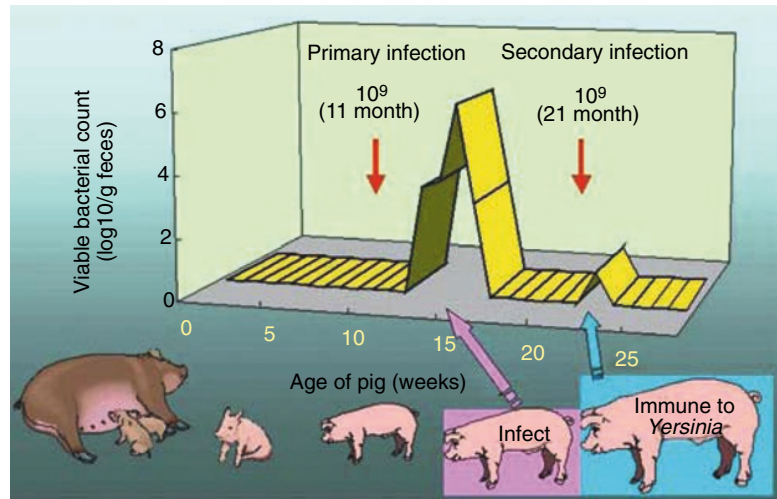


Fig. 32. Regional distribution of REAP patterns of *Y. pseudotuberculosis* serotypes O:1b and 4b among wild animals, in river water and humans in eastern Shimane prefecture.

Fig. 33. Pigs are viable carriers of *Y. enterocolitica* serotype O:3 only during primary infection. From Fukushima et al. (1984a).



humans and wild animals belonged to serotype O:1b and REAP pattern D (O:1b/D) and serotype O:4b/B, G, L, with the most predominant type being O:4b/G. The majority of serotype O:1b/D strains was prevalent among humans and wild animals in a limited area of Chugoku Mountain district of Japan. In much the same area with serotype O:1b/D strains, serotype O:4b/B strains were prevalent. Serotype O:4b/G strains were prevalent in Shimane Peninsula, while west of this area, strains of serotypes O:1b/D and O:4b/B were prevalent. Thus, serotype O:4b/G, widespread among wild animals in the examined area, seems to act as an effective barrier against other types of *Y. pseudotuberculosis*.

In eastern Japan, human and wild small rodent *Y. pseudotuberculosis* infections are rare (Saitoh et al., 1994; Hayashidani et al., 1995), while *Y. enterocolitica* serotype O:8 infection of wild rodents (2.2–5.2%; Iinuma et al., 1992; Hayashidani et al., 1995) and zoo animals (Sasaki et al., 1989) is common. In the Tsugaru region of Aomori Prefecture, where *Y. enterocolitica* serotype O:8 is highly prevalent in wild rodents in mountainous areas and cause sporadic human infections (Ichinohe et al., 1991; Saitoh et al., 1994), *Y. pseudotuberculosis* is infrequently found in wild animals (Hayashidani et al., 1995).

In the investigation of *Yersinia* in 1200 slaughtered pigs from 96 pig farms in Shimane Prefecture (Fukushima et al., 1989b; Fukushima et al., 1990b), pigs on 15 (6.4%) and 6 (1.6%) farms were found infected with *Y. enterocolitica* serotype O:3 and *Y. pseudotuberculosis* serotypes O:1b, 3, or 4b, respectively (Table 27). With the exception of the pig infections on two farms, pig infections were due either to *Y. pseudotuberculosis* or to *Y. enterocolitica* but never to both bacteria in each farm. The period of primary

infection is the only time when pigs are carriers of viable *Yersinia* (Fukushima et al., 1984a; Fig. 33).

In two remote mountainous villages of Okayama Prefecture (Inoue et al., 1988), two community infections with *Y. pseudotuberculosis* prevailed among residents who drank unchlorinated well water or mountain stream water (Fig. 34). This infection affected 173 of 245 children (70.6%) and 87 of 1157 adult residents (7.5%) in one outbreak, and 11 of 17 children (64.7%) in another outbreak, in which adult residents were not affected.

**CONCLUSION** *Yersinia pseudotuberculosis* has a wide distribution in most countries with cold climates and has been recognized as an important causal agent of sporadic and epidemic human enteric disease (Mair, 1965; Tsubokura et al., 1989). An interesting feature of the occurrence of *Y. pseudotuberculosis* infections in Japan, as compared to that in Western countries, is that 94 outbreaks involving 4741 patients throughout Japan from 1927 to 1950, and Kawasaki syndrome have occurred among children up until the 1990s (Fukushima et al., 1985b; Sato, 1987a; Tsubokura et al., 1989; Toyokawa et al., 1993). Almost all cases of *Y. pseudotuberculosis* infections in Japan occurred in mountainous areas, where residents drank natural unchlorinated water contaminated by *Y. pseudotuberculosis*-infected wild animals. Recently, waterworks and a decrease in child population in remote mountainous areas of Japan led to a gradual reduction in the importance of this bacterium in human infections. Therefore, the epidemiological importance of *Y. pseudotuberculosis* will decrease and will be replaced by its importance for plague control, since the enteropathogenic yersiniae,

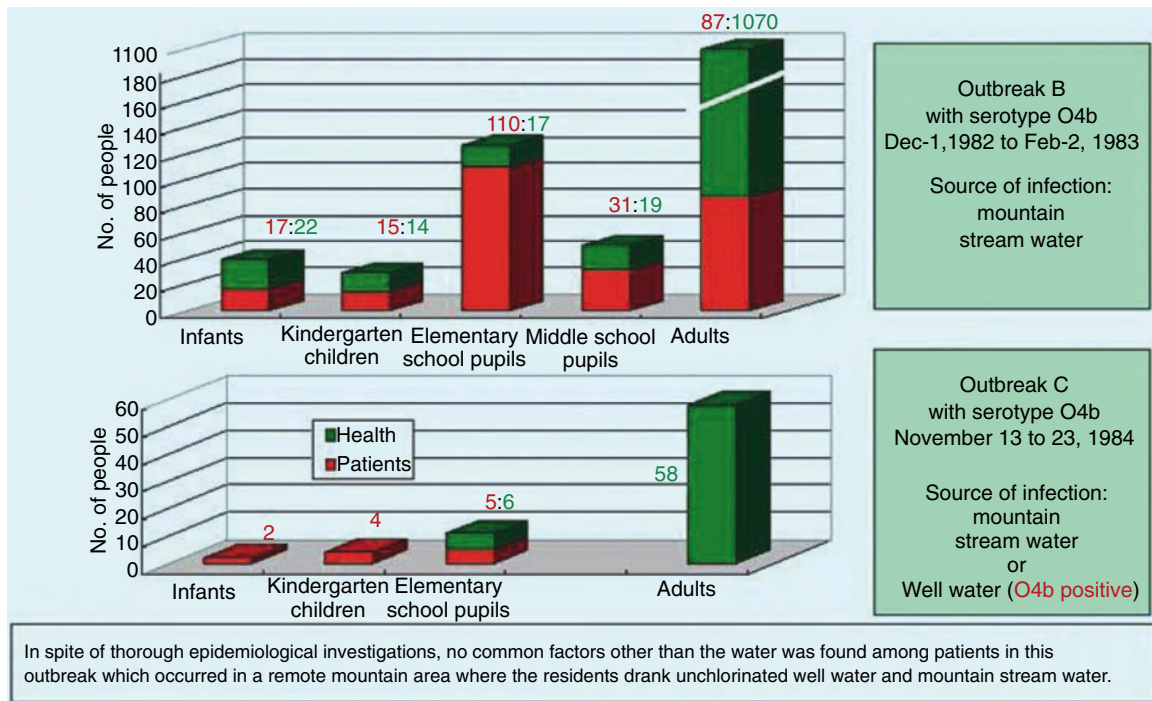


Fig. 34. Two outbreaks in villages in Okayama Prefecture. From Inoue et al. (1988).

which are widespread among mammalian populations in various parts of the world, might serve as an efficient barrier against *Y. pestis* spread from natural foci (Alonso et al., 1980).

### Clinical Manifestations of *Y. pseudotuberculosis* Infections (Mikael Prentice)

**GASTROINTESTINAL ILLNESS** *Yersinia pseudotuberculosis* causes mesenteric adenitis and chronic diarrhea in a wide variety of animals and birds on all continents (Mair, 1965; Martins et al., 1998). Outbreaks are noted in zoo animals (Bielli et al., 1999) and farmed deer (Sanford, 1995). Infection usually resolves spontaneously, but in stressful conditions in young animals, may progress to a fatal septicemia with widespread deposition of caseating lesions in lymphatic tissue (Sanford, 1995). In humans, the usual presentation includes fever and abdominal pain followed by diarrhea and vomiting (Knapp, 1958; Van Noyen et al., 1995). As with *Y. enterocolitica*, central abdominal and right iliac fossa pain that may simulate appendicitis (pseudoappendicitis) occurs (Weber et al., 1970; El-Maraghi and Mair, 1979; Attwood et al., 1987). At laparotomy, the usual finding is mesenteric adenitis without appendicitis and occasionally inflammation of the terminal ileum and cecum (Weber et al., 1970; Attwood et al., 1987; Physiopathology). In some infected children, enlargement of mesen-

teric lymph nodes sufficient to create an abdominal mass may be seen (Jelloul et al., 1997) and intussusception can occur (Koo et al., 1996a). Occasionally, *Y. pseudotuberculosis* has been isolated from an inflamed appendix (true appendicitis; Tsubokura et al., 1973; Grant et al., 1994) and has been associated with the rare chronic syndrome of granulomatous appendicitis (Lamps et al., 2001).

Histology of affected nodes shows granulomas with central necrosis and microabscess formation (Weber et al., 1970; El-Maraghi and Mair, 1979), and the organism can be recovered from mesenteric node culture (Weber et al., 1970; El-Maraghi and Mair, 1979). Isolation from feces is much less common than for *Y. enterocolitica* (Mair, 1965; Mair and Fox, 1986; Van Noyen et al., 1995), possibly because of poor growth on enteric media on primary isolation (Mair and Fox, 1986) and in most cases diagnosis is by serology (Mair and Fox, 1986; Attwood et al., 1987).

**SYSTEMIC DISEASE Septicemia** As for *Y. enterocolitica*, *Y. pseudotuberculosis* septicemia often affects patients with a pre-existing condition such as diabetes or a condition involving iron overload and/or liver disease (Mollaret et al., 1964; Marlon et al., 1971) including hemochromatosis (Conway et al., 1989), sickle cell disease (Bradley and Skinner, 1974), thalassemia (Gordts et al., 1984) or chronic hemodialysis (Boelaert et al., 1987). Unlike mesenteric aden-

itis, septicemia presentation is associated with a high mortality (Knapp, 1958; Macaulay et al., 1967; Borowski et al., 1971; Marlon et al., 1971; Ljungberg et al., 1995). In one case report of a patient with adult onset diabetes mellitus and *Y. pseudotuberculosis* septicemia, death occurred despite low levels of circulating endotoxin and cytokines normally associated with serious Gram-negative sepsis (Valtonen et al., 1995). Unlike *Y. enterocolitica*, *Y. pseudotuberculosis* septicemia associated with transfusion of stored blood has not been reported.

**Mucocutaneous Syndromes** *Yersinia pseudotuberculosis* has been implicated in the etiology of two childhood systemic syndromes: Izumi fever, first seen after the Second World War in Japan (Sato et al., 1983), and a similar syndrome first described in Vladivostok in 1959 termed “Far Eastern Scarletiform Fever” (Kuznetsov, 1974). These involved fever, rash, diarrhea, skin desquamation, strawberry tongue, vomiting, red and cracked lips, abdominal pain, arthralgias, and conjunctivitis. There is a case report of a similar illness in the child of an American serviceman in Korea (Krober et al., 1983). These symptoms and signs overlap with Kawasaki disease (Chiba et al., 1983; Sato et al., 1983), an acute febrile illness in infants and children worldwide carrying a 20% risk of developing coronary artery aneurysms (American Heart Association, 2001). *Yersinia pseudotuberculosis* infection has been described in one case report of Kawasaki disease in Japan (Konishi et al., 1997) and Kawasaki disease criteria have been found in cases of *Y. pseudotuberculosis* infection in Japanese children (Baba et al., 1991). The cause of Kawasaki disease is unknown but an immunological process involving bacterial superantigens has been suggested (Yarwood et al., 2000; Superantigens of *Y. pseudotuberculosis*). Superantigen (*Y. pseudotuberculosis*-derived mitogen, YPM) production occurs in *Y. pseudotuberculosis* infection (Abe et al., 1997b), but there is no evidence linking *Y. pseudotuberculosis* with most clinical cases of Kawasaki disease (Yanagawa, 1989; Uchiyama and Kato, 1999).

**POST-INFECTIVE PHENOMENA** *Yersinia pseudotuberculosis* infection is a significant cause of childhood interstitial nephritis in Japan (Iijima et al., 1989; Kobayashi et al., 2000) and Korea (Koo et al., 1996b). However, this syndrome of acute tubulointerstitial nephritis (interstitial edema with inflammatory cell infiltrates and tubular damage) is a rarer cause of childhood renal failure than glomerulonephritis even in these countries (Kobayashi et al., 2000). There is a report of *Y. pseudotuberculosis* infection and nephritis in an adult in Finland (Tertti et al., 1984). Hemolytic

uremic syndrome has been reported in association with *Y. pseudotuberculosis* infection in two adults in the United Kingdom (Davenport et al., 1987; Davenport and Finn, 1988) and a child in California (Prober et al., 1979).

The other main postinfective complication of *Y. pseudotuberculosis* infection is arthritis (Yli-Kerttula et al., 1995; Clinical manifestations of *Y. enterocolitica* and Immunology). In one patient, this has been associated with persistence of bacterial antigens and *Y. pseudotuberculosis* 16S rRNA detectable by reverse transcriptase-polymerase chain reaction (RT-PCR) in affected joints over several years (Gaston et al., 1999). HLA-B27-negative individuals are subject to reactive arthritis following *Y. pseudotuberculosis* infection (Tertti et al., 1984) but seem less likely to develop ankylosing spondylitis than HLA B27-positive individuals (Yli-Kerttula et al., 1995). In the long term (over 10 years), arthritis may persist despite the disappearance of detectable circulating anti-*Y. pseudotuberculosis* antibodies (Yli-Kerttula et al., 1995). The numbers of individuals suffering reactive arthritis may depend on the infecting strain (Tertti et al., 1989; Yli-Kerttula et al., 1995). As for reactive arthritis following infection with *Y. enterocolitica* and other bacteria, erythema nodosum, uveitis and keratoderma often occur as part of the presentation of *Y. pseudotuberculosis*-associated reactive arthritis (Tertti et al., 1984; Tertti et al., 1989; Yli-Kerttula et al., 1995).

### Bacteriological Diagnosis of *Y. pseudotuberculosis* (Georges Wauters)

*Yersinia pseudotuberculosis* is able to grow on most selective media used for *Y. enterocolitica* (Bacteriological diagnosis of *Y. enterocolitica*) but its growth is often poor and delayed. Cold enrichment is suitable as for *Y. enterocolitica*.

*Yersinia pseudotuberculosis* can be differentiated from *Y. pestis* by motility and the presence of urease, from *Y. enterocolitica* by the lack of ornithine-decarboxylase, by acid production from rhamnose and usually melibiose, but not from sucrose and cellobiose (Table 1).

Thirty somatic antigens and five H antigens have been described in *Y. pseudotuberculosis* but a simplified antigenic scheme containing 14 serogroups has been proposed by Tsubokura and Aleksic (Tsubokura and Aleksic, 1995; Epidemiology of *Y. pseudotuberculosis*).

### Pathogenesis

One critical element for the expression of a pathogenicity phenotype in *Yersinia* is the presence of the virulence plasmid (pYV). Without

this replicon, yersiniae are avirulent. In addition, the subgroup of highly pathogenic strains possesses a chromosomal entity, the high pathogenicity island (HPI), which is required for their systemic dissemination in the host. These two large genetic elements, although crucial for the development of disease (mild or severe), are necessary but not sufficient to cause an infection. Many other factors, including invasins, toxins, and adhesins, participate in the pathogenic potential of yersiniae. The best-characterized, confirmed or putative, virulence factors of enteropathogenic *Yersinia* are described in this chapter.

### The pYV Virulence Plasmid (Guy Cornelis)

#### DEVICES TO INJECT BACTERIAL PROTEINS ACROSS EUKARYOTIC CELL MEMBRANES

*A Plasmid Determining Virulence* The three pathogenic *Yersinia* species (*Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*) are known since the early 1960s to be dependent on  $\text{Ca}^{2+}$  for their growth at 37°C. Variants that lost this requirement appear spontaneously when wild-type bacteria are plated at 37°C in the absence of  $\text{Ca}^{2+}$ . These variants, called “ $\text{Ca}^{2+}$ -independent” have lost their virulence (Higushi and Smith, 1961). This  $\text{Ca}^{2+}$ -dependency phenotype offered an extraordinary clue to the pathogenicity arsenal because nonvirulent mutants could be easily detected and even selected for. In 1980, it appeared that both virulence and  $\text{Ca}^{2+}$ -dependency were encoded by a 70-kb plasmid (Zink et al., 1980; Gemski et al., 1980; Portnoy and Falkow, 1981a). Under conditions of growth restriction, it then appeared that this plasmid governs the synthesis of a set of about 12 proteins called “Yops” for “*Yersinia* outer membrane proteins” (Portnoy et al., 1981b; Straley and Brubaker, 1981; Bölin et al., 1982; Martinez, 1983; Cornelis et al., 1986; Forsberg et al., 1987). The LcrV protein, an antigen of *Y. pestis*, discovered in the mid 1950s (Burrows and Bacon, 1956), turned out to be one of these Yops (Straley and Brubaker, 1981). Although initially described as outer membrane proteins, the Yops could also be recovered from the culture supernatant (Heesemann et al., 1984), and they turned out later to be actually secreted proteins (Michiels et al., 1990). Their secretion appeared to occur by a new pathway (now called “type III”) and to require a specific apparatus of a new type, now called the “Ysc apparatus,” which is also encoded by the pYV plasmid (Michiels et al., 1991).

*The Yersinia Ysc Secretion Apparatus* “Yop secretion” was discovered around 1990 while trying to understand the mysterious phenomenon of  $\text{Ca}^{2+}$ -dependency: when incubated at 37°C in the

absence of  $\text{Ca}^{2+}$ , *Yersinia* bacteria do not grow, but instead, release large amounts of proteins called Yops in the culture supernatant (Michiels et al., 1990). Although it is generally referred to as Yop “secretion,” it is not a physiological secretion but rather a massive leakage resulting from the artificial opening of an otherwise tightly controlled delivery apparatus. Despite that it is presumably artefactual, this observation turned out to be of paramount importance because it allowed the characterization of the Ysc (for Yop secretion) secretion apparatus, an archetype for the type III secretion systems. The Ysc apparatus, also referred to as the “Ysc injectisome” (see below), has 29 Ysc proteins. Ten of these Ysc proteins (YscD, -J, -L, -N, -Q, -R, -S, -T, -U and -V) have counterparts in almost every type III secretion apparatus as well as in the flagellum. Their homologues in the flagellum are localized in the MS ring and the C ring, which constitute the inner part of the basal body (Minamino and Macnab, 1999). In good agreement with this finding, YscD, -R, -U and -V, (formerly called “LcrD”) have been shown and YscS and -T have been predicted to span the inner membrane. YscN has ATP-binding motifs (Walker boxes A and B) resembling the b catalytic subunit of the F<sub>0</sub>F<sub>1</sub> proton translocase and related ATPases (Woestyn et al., 1994). Also, YscN is part of a complex including also YscL, YscK and YscQ (Jackson and Plano, 2000). YscJ is a lipoprotein that has not been localized yet, but its counterpart in *P. syringae* has been shown to span the inner and the outer membranes (Deng and Huang, 1999). Thus, the ten conserved proteins constitute the proximal part of the injectisome, spanning or attached to the inner membrane.

YscC also has counterparts in every type III secretion system but not in the flagellum. It belongs to the family of secretins, a group of outer-membrane proteins involved in the transport of various macromolecules and filamentous phages across the outer membrane. As do the other secretins, it forms a ring-shaped structure with an external diameter of about 200 Å and an apparent central pore of about 50 Å (Koster et al., 1997). At least one disulfide bond is essential for its assembly (Jackson and Plano, 1999), and its proper insertion in the outer membrane requires the presence of an ancillary lipoprotein called “YscW” (Koster et al., 1997).

Some Ysc proteins including YscO, -P and -X are released by  $\text{Ca}^{2+}$ -chelation together with the substrates of the Ysc apparatus. They are nevertheless required for Yop secretion and hence must constitute the most external part of the injectisome (Payne and Straley, 1998a; Payne and Straley, 1999; Iriarte and Cornelis, 1999a; Day and Plano, 2000a; Stainier et al., 2000). The fact that some of the Ysc proteins are themselves



secreted upon  $\text{Ca}^{2+}$ -chelation suggests that some of the Yop proteins could also be proteins of the injectosome structure rather than substrates as anticipated. This could be the case of YopN. Indeed, it is encoded in the same locus as *yscX* and it is required to prevent a deregulated secretion of Yops (Forsberg et al., 1991).

According to electron microscopy observations, the Ysc injectosome ends up with a needle formed by polymerization of the 6-kDa YscF (Hoiczuk and Blobel, 2001). Isolated needles have a length of 600–800 Å and a width of 60–70 Å. The needle has a hollow center of about 20 Å.

Little is known about the actual mechanism of export, but it is generally assumed that the Ysc apparatus serves as a hollow conduit through which the exported proteins travel to cross the two membranes and the peptidoglycan barrier in one step. Whether proteins travel folded or unfolded has not yet been demonstrated, but given the size of channel, it is likely that they travel, at least partially, unfolded.

*Translocation of Effectors Across Eukaryotic Cell Membranes* Purified secreted Yops have no cytotoxic effect on cultured cells, although live extracellular *Yersinia* have such an activity. Cytotoxicity was nevertheless found to depend on the capacity of the bacterium to secrete YopE and YopD. However, YopE alone was found to be cytotoxic when microinjected into the cells. This observation led to the hypothesis that YopE is a cytotoxin that needs to be injected into the eukaryotic cell's cytosol by a mechanism involving YopD to exert its effect (Rosqvist et al., 1991). In 1994, this hypothesis was demonstrated by two different approaches. The group of Hans Wolf-Watz used immunofluorescence and confocal laser scanning microscopy examinations (Rosqvist et al., 1994), while the group of Guy Cornelis introduced a reporter enzyme strategy based on the calmodulin-activated adenylate cyclase (Sory and Cornelis, 1994). Infection of a monolayer of eukaryotic cells by a recombinant *Y. enterocolitica* producing a hybrid protein made of the N-terminus of YopE and the catalytic domain of the adenylate cyclase of *Bordetella pertussis* (YopE-Cya protein) led to an accumulation of cyclic AMP in the cells. Since the cyclase is not functional in the bacterial cell and in the culture medium because of a lack of calmodulin, this accumulation of cAMP signified the internalization of YopE-Cya into the cytosol of eukaryotic cells (Sory and Cornelis, 1994). Thus, extracellular *Yersinia* inject YopE into the cytosol of eukaryotic cells by a mechanism that involves at least one other Yop protein, YopD. That YopH was also injected into the target cell cytosol was later demonstrated (Persson et al., 1995; Sory et al., 1995), and the requirement of

YopB for delivery of YopE and YopH, like YopD was shown (Boland et al., 1996; Nordfelth and Wolf-Watz, 2001). These observations led to the present concept that Yops are a collection of intracellular effectors (including YopE and YopH) and proteins (including YopB and YopD) required for translocation of these effectors across the plasma membrane of eukaryotic cells (Cornelis and Wolf-Watz, 1997). Delivery of effector Yops into eukaryotic cells appears to be a directional phenomenon in the sense that the majority of the Yop effector molecules produced are directed into the cytosol of the eukaryotic cell and not to the outside environment (Rosqvist et al., 1994; Persson et al., 1995).

This model of intracellular delivery of Yop effectors by extracellular adhering bacteria is now largely supported by a number of other results, including immunological observations. While antigens processed in phagocytic vacuoles of phagocytes are cleaved and presented by major histocompatibility complex (MHC) class II molecules, epitope 249–257 of YopH produced by *Y. enterocolitica* during a mouse infection is presented by MHC class I molecules, like cytosolic proteins (Starnbach and Bevan, 1994).

*A Pore Formed by Translocators* The translocators YopB and YopD have hydrophobic domains suggesting that they could act as transmembrane proteins (Rosqvist et al., 1994; Sory and Cornelis, 1994; Persson et al., 1995; Boland et al., 1996). In agreement with this, *Yersinia* has a contact-dependent lytic activity on sheep erythrocytes, depending on YopB and YopD (Hakansson et al., 1996; Neyt and Cornelis, 1999a), which suggests that the translocation apparatus involves some kind of a pore in the target cell membrane by which the Yop effectors pass through to reach the cytosol. This YopB- and YopD-dependent lytic activity is higher when the effector *yop* genes are deleted, suggesting that the pore is normally filled with effectors (Hakansson et al., 1996; Neyt and Cornelis, 1999a). The idea of a translocation pore is further supported by the observation that the membrane of macrophage-like cells infected with an effector polymutant *Y. enterocolitica* becomes permeable to small dyes (Neyt and Cornelis, 1999a). If the macrophages are pre-loaded prior to the infection with a low-molecular weight fluorescent marker, they release the fluorescent marker but not cytosolic proteins, indicating that there is no membrane lysis but rather insertion of a small pore (diameter 16–23 Å) into the macrophage plasma membrane (Neyt and Cornelis, 1999a). The hypothesis of a channel is reinforced by the observation that artificial liposomes that have been incubated with *Yersinia* contain channels detectable by electrophysiology (Tardy et al.,

1999). All these events are dependent on the presence of the translocators YopB and YopD. These two hydrophobic Yops seem thus to be central for the translocation of the effectors and for the formation of a channel in lipid membranes. They presumably play different roles in pore formation. Indeed, YopB alone can disturb artificial membranes, while YopD cannot. Moreover, YopD has been shown to end up in the cytosol of eukaryotic cells (Francis and Wolf-Watz, 1998).

YopB and YopD are encoded by a large operon that also encodes LcrV, LcrG and the chaperone SycD. Also, LcrV is a secreted Yop that has a different name for historical reasons. The fact that LcrG and LcrV are encoded together with translocators suggests that they could also be involved in the translocation step. Not surprisingly, LcrV interacts with YopB and YopD (Sarker et al., 1998), is surface-exposed before target cell contact (Pettersson et al., 1999), and is indeed required for translocation (Sarker et al., 1998; Lee et al., 2000; Holmstrom et al., 2001). However, in addition to this role as a translocator, LcrV also plays a still not understood intrabacterial regulatory role in Yop synthesis (Nilles et al., 1998).

Unlike YopB, YopD and LcrV, LcrG is not a released protein but its exact localization in the bacterium remains elusive. It is required for efficient translocation of *Yersinia* Yop effector proteins into the eukaryotic cells, but it is not required for pore formation. It binds to LcrV. Like LcrV and presumably through its interaction with LcrV, it also plays a regulatory role (Lee et al., 2000). LcrG has also been shown to bind heparin sulfate proteoglycans (Boyd et al., 1998) but the significance of this binding is not clear.

The fact that LcrV, YopB and YopD are involved in translocation of the effector Yops does not necessarily exclude that some of them could themselves end up in the eukaryotic cytoplasm, as was shown for YopD and LcrV (Francis and Wolf-Watz, 1998; Fields and Straley, 1999).

It is not known yet whether the translocators are already present at the tip of the “needle” prior to contact with a eukaryotic cell or whether they are the first proteins to be secreted upon contact with a target cell.

*The Cytosolic Chaperones* A hallmark of type III secretion is that normal secretion of some substrate proteins requires the presence of small cytosolic chaperones of a new type (Menard et al., 1994; Wattiau et al., 1994b; Wattiau et al., 1996). Generally, these chaperones are encoded by genes proximal to the gene encoding the protein they serve, and this is a useful way to recognize such chaperones. However, in *Y. pseudotuberculosis*, the gene encoding the chap-

erone of YopH was separated from the *yopH* gene by a large inversion. SycE, the chaperone of YopE is the archetype of a first family of “type III chaperones” (Wattiau and Cornelis, 1993). There are four typical representatives of this family in *Yersinia*: SycE, SycH, SycT and SycN. One could also add to this list the less typical YscB, acting as a co-chaperone for YopN (Day and Plano, 1998; Jackson et al., 1998). All these chaperones are small (14–15 kDa) proteins with a putative C-terminal amphiphilic  $\alpha$ -helix, and most of them are acidic (pI 4.4–5.2). They specifically bind only to their partner Yop, and in their absence, secretion of the cognate protein is severely reduced, if not abolished. However, the exact role of these chaperones remains elusive. Both SycE and SycH bind to their partner Yop at a unique site spanning roughly residues 20 to 70 (Sory et al., 1995). Surprisingly, when this site is removed, the cognate Yop is still secreted—though maybe in reduced amounts—and the chaperone becomes dispensable for secretion (Woestyn et al., 1996). This suggests that it is the binding site itself that creates the need for the chaperone and thus the chaperone somehow protects this site from premature associations, which would lead to degradation. In agreement with this hypothesis, SycE has indeed an anti-degradation role: the half-life of YopE is longer in wildtype bacteria than in *sycE* mutant bacteria (Fritz-Lindsten et al., 1995; Cheng et al., 1997). In addition to having this putative role of body-guard, SycE also acts as a secretion pilot leading the YopE protein to the secretion locus (see below). Finally, both SycE and SycH are required for efficient translocation of their partner Yop into eukaryotic cells (Sory et al., 1995). However, when YopE is delivered by a *Yersinia* polymutant strain that synthesizes an intact secretion and translocation apparatus but no other effector, it appears that YopE is delivered without the chaperone and the chaperone-binding site (Boyd et al., 2000). Thus, the SycE chaperone appears to be needed only when YopE competes with other Yops for delivery. This suggests that the Syc chaperones could be involved in some kind of a hierarchy for delivery. This new hypothesis about the role of the Syc chaperones fits quite well with the observation that only a subset of the effectors seem to have a chaperone. Little is known about the role of SycT and SycN. However, there is an unexpected complexity for the latter: SycN apparently requires YscB working as a co-chaperone (Day and Plano, 1998).

SycD is the archetype of a second group of “type III chaperones.” It serves the translocators YopB and YopD (Wattiau et al., 1994b; Neyt and Cornelis, 1999b), and in its absence, YopD and YopB are less detectable inside the bacterial cell.

SycD appears to be different from SycE and SycH in the sense that it binds to several domains on YopB (which evokes rather SecB, a molecular chaperone in *E. coli* dedicated to the export of newly synthesized proteins) and also has multiple binding sites on its targets (Fekkes and Driessen, 1999). IpgC, the related chaperone from *S. flexneri*, has been shown to prevent the intrabacterial association between translocators IpaB and IpaC (Menard et al., 1994). The similarity between IpgC and SycD suggested that SycD could play a similar role and would thus prevent the intrabacterial association of YopB and YopD. However Neyt and Cornelis (1999b) observed that intrabacterial YopB and YopD are associated in the bacterium, even in the presence of SycD. Since YopB and YopD have also the capacity to bind to LcrV, one could speculate that SycD prevents a premature association, not between YopB and YopD but rather between YopB, YopD and LcrV. However, this has not been shown yet.

Finally, it appears that some external components of the injectisome itself have a chaperone. YscG has been suggested to be the chaperone of YscE (Day et al., 2000b) and YscY could be the chaperone of YscX (Day and Plano, 2000a). The role of these chaperones could be to prevent intrabacterial association of the distal components of the injectisome.

*Recognition of the Transported Proteins* Effectors delivered by the type III secretion systems have no classical cleaved N-terminal signal sequence (Michiels et al., 1990). However, it is very clear that Yops are recognized by their N-terminus but that no classical signal sequence is cleaved off during Yop secretion (Michiels et al., 1990). The minimal region shown to be sufficient for secretion was gradually reduced to 17 residues of YopH (Sory et al., 1995), to 15 residues of YopE (Sory et al., 1995), and to 15 residues of YopN (Anderson and Schneewind, 1997).

The secretion domains of the Yops are not similar, suggesting recognition of a conformational motif of the nascent protein (Michiels et al., 1990). To explain how the same secretion apparatus could recruit proteins with no common signal (Wattiau and Cornelis, 1993), it was suggested that the Syc chaperones could serve as pilots. However, this hypothesis was questioned when it appeared that YopE could be secreted even if its chaperone-binding domain had been deleted (Woestyn et al., 1996). It was then concluded that secretion was only dependent on the short N-terminal signal, but secretion of a Yop lacking only this N-terminal signal had never been tested.

A systematic mutagenesis of the secretion signal by Anderson and Schneewind (Anderson

and Schneewind, 1997; Anderson and Schneewind, 1999) led to doubts about the proteic nature of this signal. No point mutation could be identified that specifically abolished secretion of YopE, YopN and YopQ. Moreover, some frameshift mutations that completely altered the peptide sequences of the YopE and YopN signals also failed to prevent secretion. Anderson and Schneewind (Anderson and Schneewind, 1997; Anderson and Schneewind, 1999) concluded from these observations that the signal that leads to the secretion of these Yops could be in the 5'-end of the messenger RNA, rather than in the peptide sequence. Translation of *yop* mRNA might be inhibited by either its own RNA structure or as a result of its binding to other regulatory elements. However, this mRNA signal hypothesis is contradicted by recent work of Lloyd and colleagues (Lloyd et al., 2001). These authors show that mutations of the first 11 codons of YopE, modifying the mRNA but not the amino acid sequence, do not impair secretion, suggesting that it is the N-terminus of YopE and not the 5' end of *yopE* mRNA that serves as a targeting signal.

To determine whether this N-terminal (or 5'-terminal) signal is absolutely required for YopE secretion, Cheng et al. (1997) deleted codons 2–15 and they observed that 10% of the hybrid proteins deprived of the N-terminal secretion signal were still secreted. They inferred that there is a second secretion signal, and they showed that this second, and weaker, secretion signal corresponds to the SycE-binding site. Not surprisingly, this secretion signal is only functional in the presence of the SycE chaperone (Cheng et al., 1997), rejuvenating the pilot hypothesis of Wattiau and Cornelis (1993). The Syc chaperone could ensure stability and proper conformation of the protein and target it to the secretion channel. At the moment of secretion, the chaperone must be released from the partner Yop to allow secretion.

Thus, the effectors that have a chaperone, like YopE, YopH, YopN and YopT, are likely to have two secretion signals that operate during *in vitro* secretion: one linked to translation and one, post-translational. What is the relative importance of these two systems *in vivo* remains to be elucidated.

*Control of the Injection* We have seen that “type III secretions” can secrete their substrate *in vitro* under given conditions, such as Ca<sup>2+</sup>-chelation for instance. What is the triggering signal *in vivo*? Most probably, it is contact with a eukaryotic cell. Several reports on *Yersinia* have shown that Yops delivery is a “directional” phenomenon in the sense that most of the load is delivered inside the eukaryotic cell and that there is little leakage

(Persson et al., 1995). With the assays used, measurement of the degree of “directionality” is somewhat discrepant (Boland et al., 1996), but there is no doubt that the majority of the released Yops load ends up within the eukaryotic cell and thus that contact must be the signal. Pettersson et al. (1996) provided a nice visual demonstration of the phenomenon. By expressing luciferase under the control of a *yop* promoter, they showed indeed that active transcription of *yop* genes is limited to bacteria that are in close contact with eukaryotic cells.

**EFFECTOR PROTEINS AND HOST RESPONSES** *The Cytoskeleton is a Target of YopE, YopH, YopT and YpkA (YopO)* Four effectors, out of six identified so far, exert a negative role on the cytoskeleton dynamics, and by doing so, contribute to the strong resistance of *Yersinia* to phagocytosis by macrophages (Rosqvist et al., 1991; Grosdent and G. R. Cornelis, unpublished observation). YopH is a powerful phosphotyrosine phosphatase resembling eukaryotic phosphatases. The catalytic activity is exerted by the C-terminal domain (residues 206–408), which contains a phosphate-binding loop including a critical cysteine residue (cys 403; Guan and Dixon, 1993). In HeLa cells, YopH dephosphorylates p130<sup>Cas</sup>, paxillin and the focal adhesion kinase (FAK; Black and Bliska, 1997; Persson et al., 1997; Black et al., 1998), which leads to disruption of the focal adhesion and a reduced invasin-mediated engulfment. YopH is specifically targeted to the focal complexes and residues 223–226, known to be surface-exposed and involved in this process. Deletion of these targeting residues affects the antiphagocytic effect. These observations also apply to the J774 macrophage-monocyte cell line, at least in the absence of opsonization (Persson et al., 1999). In the latter cell line, a catalytically inactive YopH co-precipitates not only with p130<sup>Cas</sup> but also with the Fyn-binding protein FYB (Hamid et al., 1999) and the SKAP-HOM protein (Black et al., 2000). The N-terminal domain of YopH binds not only to SycH (Sory et al., 1995) but also the substrate (Montagna et al., 2001). Yao et al. (1999) observed that T and B cells transiently exposed to *Yersinia* are impaired in their ability to be activated through their antigen receptors. The T cells are inhibited in their ability to produce cytokines, and B cells are unable to upregulate surface expression of the costimulatory molecule B7.2, in response to antigenic stimulation. This block of activation results from the inhibition of early phosphorylation events (Yao et al., 1999). Through the analysis of various mutants, YopH appeared to be the main effector involved in these events. Thus YopH not only contributes to the evasion of the innate immune response by inhib-

iting phagocytosis but it can also incapacitate the host adaptive immune response.

YopE has been known for a long time to disrupt actin filaments (Rosqvist et al., 1990; Rosqvist et al., 1991). In vitro, it acts as a GTPase-activating protein (GAP) for monomeric GTPases of the Rho family controlling the cytoskeleton dynamics (von Pawel-Rammingen et al., 2000). YopE has an arginine finger motif similar to those found in other GAP proteins. Exchange of arginine 144 from this motif results in loss of the GAP activity and inability to induce cytotoxicity on cultured HeLa cells (von Pawel-Rammingen et al., 2000). Experiments conducted in human umbilical vein endothelial cells (HUVECs) show that YopE can inhibit Rac- but not Rho- or Cdc42-regulated actin structures, and more specifically, that YopE is capable of blocking Cdc42-dependent Pac activation but not direct Pac activation (Andor et al., 2001). This GAP activity of YopE has been shown to be a key of the antiphagocytic function of YopE (Black et al., 2000).

YopT exerts its depolymerizing effect on actin (Iriarte and Cornelis, 1998) by modifying RhoA, a GTPase that regulates the formation of stress fibers (Zumbihl et al., 1999). The exact nature of the modification is still unknown.

YpkA (YopO) also leads to a morphological alteration of the target cells due to the disruption of the actin cytoskeleton. A serine-threonine kinase, YpkA, shows some similarity with eukaryotic serine/threonine protein kinases (Galyov et al., 1993) and becomes activated by interacting with actin through the C-terminal twenty amino acids of YpkA (Juris et al., 2000). The amino terminus contains the kinase domain, while the carboxy terminus interacts with RhoA and Rac-1, preferably bound to GDP (Barz et al., 2000; Dukuzumuremyi et al., 2000). Inside the HeLa cells, the YpkA protein is targeted to the inner surface of the plasma membrane (Hakansson et al., 1996).

*YopP/YopJ Downregulates the Inflammatory Response* YopP/YopJ is a key player in the downregulation of the inflammatory response that is observed during *Yersinia* infection. In vitro, YopP/YopJ has indeed been shown to counteract the normal proinflammatory response of various cell types. Its injection reduces the release of tumor necrosis factor (TNF- $\alpha$ , Boland and Cornelis, 1998a) by macrophages and of interleukin (IL-8) by epithelial (Schulte et al., 1996; Schesser et al., 1998) and endothelial cells (Töttemayer et al., 2002). It also reduces the presentation of adhesion molecules (intercellular adhesion molecule [ICAM-1] and E-selectin) at the surface of endothelial cells (Töttemayer et al., 2002) and hence presumably

reduces neutrophil recruitment to the infection sites. All these events result from the inhibition of the activation of nuclear factor (NF- $\kappa$ B), a transcription factor known to be central in the onset of inflammation (Ruckdeschel et al., 1998; Schesser et al., 1998). The inhibition of NF- $\kappa$ B activation was recently shown to result from the inhibition by YopP/YopJ of IKK $\beta$ , a kinase that phosphorylates I $\kappa$ B, the inhibitor of NF- $\kappa$ B (Orth et al., 1999). By preventing phosphorylation of I $\kappa$ B, YopP/YopJ prevents its degradation and the translocation of NF- $\kappa$ B to the nucleus.

The inhibition of NF- $\kappa$ B activation is accompanied by a lack of activation of the mitogen-activated protein (MAP) kinases (MAPK), c-jun-N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) 1 and 2 (Ruckdeschel et al., 1997; Boland and Cornelis, 1998a; Palmer et al., 1999) that is observed upon infection of macrophages by a *Yersinia* producing YopP/YopJ. This lack of activation of the MAPK results from the inhibition of the upstream MAPK kinases (MKK; Orth et al., 1999), but direct binding of YopP/YopJ to MKKs has not been documented. Inhibition of the MAPK pathways abrogates phosphorylation of cyclic AMP response element-binding (CREB) protein, another transcription factor involved in the immune response (Meijer et al., 2000).

Last but not least, YopP/YopJ induces apoptosis in macrophages but not in other cell types (Mills et al., 1997; Monack et al., 1997). This apoptosis is accompanied by cleavage of the cytosolic protein BID, the release of cytochrome *c*, and the cleavage of caspase-3, -7 and -9 (Denecker et al., 2001). The release of cytochrome *c* and the cleavage of BID can both be inhibited by caspase inhibitors, suggesting that YopP/J interferes with a signaling pathway upstream of the mitochondria (Zhang et al., 1997). However, this signaling pathway has not been identified yet, and one may wonder whether apoptosis does not result from the loss of the anti-apoptotic factor NF- $\kappa$ B (Ruckdeschel et al., 1998; Ruckdeschel et al., 2001).

Starting from the observation that the predicted secondary structure of YopJ/P resembles that of an adenovirus protease, Orth et al. (2000) identified and mutated the critical cysteine and histidine of the putative protease catalytic site. They observed that mutations in this site affected the ability of YopJ/P to inhibit activation of the MAPK pathway, as determined by measuring extracellular ERK activity in response to treatment by the epidermal growth factor (EGF). Mutant YopJ/P has also lost the capacity to inhibit translocation of NF- $\kappa$ B to the nucleus, as induced by overexpression of a constitutively active form of mitogen-activated protein/ERK kinase kinases (MEKK), in vitro (Orth et al.,

2000). A similar mutant, generated in *Y. enterocolitica* has lost the capacity to induce apoptosis of macrophages. Thus, an intact protease catalytic site is required for YopJ/P activity, suggesting that YopP could be a protease. Orth et al. (2000) pursued the protease hypothesis, discovering a limited sequence similarity between YopJ/P and the catalytic core of the yeast ubiquitin-like protease Ulp1 that cleaves the COOH-terminus of an 11-kDa small ubiquitin-related modifier (SUMO-1) protein. SUMO-1 is the best-characterized member of a rapidly growing family of ubiquitin-like proteins that are involved in posttranslational modification. To verify whether YopJ/P could act as a SUMO-protease, Orth et al. (2000) transfected cells with either wildtype or mutated forms of YopJ/P and SUMO-1. In their system, YopJ/P reduced the cellular concentration of SUMO-1-conjugated proteins as well as the amount of free SUMO-1, while cells transfected with mutant YopJ/P did not, suggesting that indeed YopJ/P acts as a SUMO-protease. This intriguing observation raises the question of consistency with the previous findings that YopJ/P interacts with the MKKs and IKK $\beta$  pathways, preventing their phosphorylation. At first sight, the two observations may even appear to be contradictory. SUMO-1 is indeed supposed to protect I $\kappa$ B $\alpha$  from phosphorylation. Thus, by de-SUMOylating I $\kappa$ B $\alpha$ , YopJ/P should promote and not prevent its phosphorylation. To reconcile the two observations, Orth et al. (2000) propose that the MKKs or IKK $\beta$  could activate and escort YopJ/P to the signaling complex, where it would silence critical SUMO-1 conjugated proteins. This would, in turn, prevent phosphorylation of MKKs and IKK $\beta$ .

**YopM** YopM is a strongly acidic protein containing leucine-rich repeats (LRRs); although it clearly contributes to pathogenesis (Leung et al., 1990), its action and target remain unknown. It belongs to a growing family of type III effectors that has several representatives in *Shigella* (*ipaH* multigene family) and *Salmonella* (Miao et al., 1999). Early work has shown that YopM binds to thrombin suggesting that YopM could play a role as an extracellular virulence factor (Leung et al., 1990). However, delivery of YopM (like the other Yop effectors) into eukaryotic target cells has been shown (Boland et al., 1996). When inside cells, YopM has been shown to travel to the cell's nucleus by a vesicle-associated pathway (Skrzypek et al., 1998), but its action in the nucleus remains unknown.

**Regulation of Transcription of the Virulon Genes** Transcription of the *ysc* genes and the *yop* genes is thermoinduced. This thermoregulation results from the interplay between the tran-

scriptional activator VirF (LcrF in *Y. pestis* and *Y. pseudotuberculosis*) and chromatin structure. Transcription of the *yop* genes, but not the *ysc* genes, is repressed by Ca<sup>2+</sup> or by mutations in the secretion apparatus. This second regulation is a feedback inhibition mechanism exerted by the closed secretion apparatus on transcription of the genes encoding the proteins to be secreted (Cornelis et al., 1995; Straley and Perry, 1995).

VirF/LcrF is a 30.9-kDa basic protein that belongs to the AraC family of regulators (Cornelis et al., 1989; Hoe et al., 1992; Lambert de Rouvroit et al., 1992; Skurnik and Toivanen, 1992; Hoe and Goguen, 1993). It has been shown to bind to a 40-bp region localized immediately upstream from the RNA polymerase binding site on the *yopE*, *yopH*, *virC* and *lcrGVsycDyopBD* promoters (Wattiau and Cornelis, 1994a). These VirF-binding sites are located in an AT-rich region and appear either isolated or repeated in opposite orientation. They contain the 13-bp consensus sequence TTTTaGYcTtTat (Wattiau and Cornelis, 1994a). In *Y. enterocolitica*, the *virF* gene itself is strongly thermoregulated and VirF is only active at 37°C.

By analogy with the secreted anti-σ factor involved in the regulation of flagellum synthesis, Hughes et al. (1993), Kutsukake (1994) and Rimpiläinen et al. (1992) suggested that feedback inhibition could be mediated by a repressor that is normally expelled via the Yop secretion machinery. In *Y. pseudotuberculosis*, they suggested that LcrQ, a 12.4-kDa secreted protein encoded by the last gene of the *virC* locus, could be this hypothetical regulator because overproduction of this protein abolishes Yop production. In *Y. enterocolitica*, there are two homologues of LcrQ, YscM1 and YscM2 (Allaoui et al., 1995b; Stainier et al., 1997). How LcrQ or YscM1 and YscM2 work is still unknown, but their mode of action is probably indirect.

THE pYVE227 PLASMID OF *YERSINIA ENTEROCOLITICA* SEROTYPE O:9

*An Overview of the Plasmid* The map of pYVe227 from *Y. enterocolitica* W227 is given in Fig. 35 (Iriarte and Cornelis, 1999b). The circular map given in Fig. 35 highlights the genes encoding the Yop virulon and the color code classifies the genes according to the part of the virulon they encode: adherence, secretion, translocation, intracellular effectors, and control of Yop release. The more detailed linear map of the pYVe227 plasmid from *Y. enterocolitica* presented in Fig. 35 gives a clear view of the relative size of the genes and shows the most important transcriptional units.

All together the genes encoding the Yop virulon occupy about two thirds of the plasmid, with

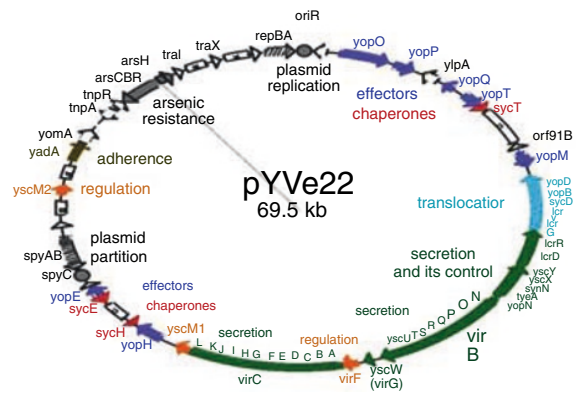


Fig. 35. Map of the pYV plasmid from *Y. enterocolitica* W227 (serotype O:9). Modified from Iriarte and Cornelis, 1999b.

only two insertion sequence (IS) elements interspersed among the *yop* and *syc* genes (Iriarte and Cornelis, 1999a). The third part of the pYVe227 plasmid, localized between the replication origin and the partition site, contains only three genes that are, or could be, involved in pathogenicity: *yadA*, and two new genes *yomA* and *pprA* (Iriarte and Cornelis, 1999a). The rest of this region consists of the replication and partition genes, the remains of a transfer operon, a transposon encoding arsenic resistance (see below), and four complete or vestigial ISs, most of which belong to the family of IS3.

*Genes Encoding the yop Virulon: a Pathogenicity Island on a Plasmid*

The secretion apparatus is composed of 29 genes organized in four main loci that are called *virA* (*yopN*, *tyeA*, *sycN*, *yscXY* and *lcrR*), *virB* (*yscNOPQRSTU*), *virG* (monocistronic, now called “*yscW*”) and *virC* (*yscABCDEFGHIJKLM*; Michiels et al., 1990; Allaoui et al., 1995a; Iriarte and Cornelis, 1999a). The loci *virB* and *virC* constitute two large operons transcribed in the same direction and separated by *yscW* and the regulatory gene *virF*. Locus *virA* is transcribed divergently, and it is not clear yet whether it also constitutes a single operon. The proteins required for translocation of the effectors across the eukaryotic cell membrane, YopB, YopD, their chaperone SycD, LcrV and LcrG, are all encoded by the same operon that is located next to *virA* and transcribed in the same orientation (Hakansson et al., 1993; Iriarte and Cornelis, 1999a). Thus, the secretion-translocation apparatus is encoded by 35 contiguous genes including the regulatory gene *virF*. The compact character of this virulence genome suggests that these genes encode some kind of a complex structure and that a long evolutionary process has been brought to completion.

At variance with the genes encoding the secretion-translocation apparatus, the genes encoding the effectors and their chaperones

appear to be more dispersed, suggesting that they were recruited more recently in the virulon. In pYVe227, the four chaperones SycE, SycH, SycD, and SycT are encoded next to their cognate gene. Gene *sycN* is separated from *yopN* by *tyeA*, but the latter also encodes a protein that interacts with YopN. Between *yopP* and *yopQ* lies the gene encoding the outer membrane lipoprotein YlpA (China et al., 1990). YlpA is highly homologous to the TraT proteins encoded by a number of bacterial plasmids.

In summary, a very compact group of 35 genes encoding the secretion-translocation apparatus is flanked on either side by the genes encoding the Yop effectors and their chaperones. All the genes encoding the virulon are thus clustered, as they would be on a pathogenicity island and virulence plasmid.

Interestingly, the gene encoding the adhesin YadA, which is essential for the Yop virulon in *Y. enterocolitica* but not in *Y. pseudotuberculosis* and *Y. pestis* is separated from the genes encoding the core of the apparatus, indicating that the adhesin may have been recruited more recently by the system (Balligand et al., 1985; Iriarte and Cornelis, 1999a).

The DNA composition of the genes encoding the type III secretion system in several bacteria differs from that of the genomic DNA of the corresponding bacteria. This is not the case here. The average G+C content of the genes encoding the Yop virulon in *Y. enterocolitica* is indeed 44.4% (Iriarte and Cornelis, 1999a), which is not very different from the reported G+C content of the *Y. enterocolitica* chromosome (46–50 mol%; Carniel et al., 1996). This observation suggests that these genes have been in Enterobacteriaceae, if not in *Yersinia*, for a long time.

*Ancillary Functions: Replication and Partition System* The replicon of the pYVe227 plasmid of *Y. enterocolitica* is of the RepFIIA type (archetype R100; Vanooteghem and Cornelis, 1990). The replication machinery consists of two proteins (the RepA replicase and the RepB regulator) and an origin of replication (*oriR*; Vanooteghem and Cornelis, 1990). The pYVe227 plasmid is incompatible with the sex factor F (Kalmykova et al., 1988) because they share a similar partition function (Biot and Cornelis, 1988). This function consists of two proteins, SpyA and SpyB, (for stability of pYV) encoded by an operon (Iriarte and Cornelis, 1999a). SpyA resembles SopA of the *E. coli* F plasmid, a 388-residue protein with a DNA-dependent ATPase activity, involved in the autoregulation of the *sopAB* operon (Mori et al., 1986). SpyB resembles SopB of the F plasmid, a DNA-binding protein that specifically binds to the F plasmid *sopC* locus (Mori et al.,

1986). Precisely 146 bp downstream from *spyB*, a sequence of 33 bp (ATTGGATATCCAGG TGACCGTGGTCCCAATTAC) is repeated twice, in the same order separated by 10 bp. Nucleotides 13 to 29 of these repeats can be aligned with the consensus sequence of the 12 directed repeats that form the partition site *sopC* of F (Mori et al., 1986; Herman and Schneider, 1992). From these similarities, it has been inferred that these repeats (called “*spyC*”) correspond to the partition site of the pYVe227 plasmid.

*An Operon Encoding Arsenic Resistance: Conquest of a New Ecological Niche* The pYVe227 plasmid of the low virulence strains of *Y. enterocolitica* (O:1,2,3, O:1,2, O:3, O:9 and O:5,27) contains a class-II transposon, Tn2502, which confers resistance to arsenite and arsenate (Neyt et al., 1997). This resistance involves four genes: three are the homologues to the *arsRBC* genes present on the *E. coli* chromosome but the fourth one, *arsH*, has no known homologue. ArsR is an arsenite-inducible transcriptional repressor, ArsB forms a transmembrane channel, and ArsC catalyzes the reduction of arsenate to arsenite, which is, in turn, expelled by the ArsB transport system. Protein ArsH could act as a regulator, even though this has never been shown. To our knowledge, this is the first example of a virulence plasmid carrying resistance genes (Neyt et al., 1997). The *ars* operon is not present on the pYVe227 plasmid of the more virulent “American” strains (serotypes O:4, O:8, O:13a, 13b, O:18, O:20 and O:21) of *Y. enterocolitica*. This suggests that the low virulence strains, which are distributed worldwide, constitute a single clone that probably emerged quite recently. At the present time, pigs represent the major reservoir of pathogenic strains of *Y. enterocolitica*, and pork meat is recognized as the major human contamination source (Tauxe et al., 1987; Lee et al., 1991; Epidemiology of *Y. enterocolitica*). Neyt et al. (1997) speculated that the *ars* transposon might have favored the establishment of a strain of *Yersinia* in pigs. Arsenic compounds were largely used before World War II as therapeutic agents to protect pigs from diarrhea caused by *Serpulina hyodysenteriae*.

*Insertion-like Sequences* The pYVe227 plasmid contains several ISs, some of which are vestigial (Iriarte and Cornelis, 1999a). Most of these belong to the IS3 family and they resemble IS222 of *Pseudomonas aeruginosa*.

THE 70-KB PLASMID OF *Y. PSEUDOTUBERCULOSIS* AND *Y. PESTIS* AND EVOLUTIONARY ASPECTS *An Overview* The virulence plasmid of *Y. pestis* has also been completely sequenced by two different

groups (Hu et al., 1998b; Perry et al., 1998). The sequence of the *Y. pseudotuberculosis* pIB1 has not been published yet but most of it is known (Persson et al., 1995). In general, the 70-kb plasmid is quite well conserved in the three species (Portnoy and Falkow, 1981a; Portnoy et al., 1981b; Biot and Cornelis, 1988; Hu et al., 1998b; Perry et al., 1998; Iriarte et al., 2002). All the 35 genes of the secretion-translocation apparatus are present in the very same order. The same effector genes are also present on the three plasmids, but they have been reshuffled. The ancillary functions of replication and partition of the three plasmids are also the same, indicating that they derive from a common ancestor.

**Major Inversions** The order of the genes is identical in pCD1 and pIB1, reinforcing the idea that *Y. pestis* and *Y. pseudotuberculosis* are extremely close. In contrast, half of the plasmid is inverted in pYVe227 (Biot and Cornelis, 1988). The inversion occurred between *yopH* and *sycH* on one side and between *ylpA* and *yopJ/yopK* on the other side. In addition, within the inverted half, the region containing the partition system, *yopE*, *sycE* and *sycH* also suffered an inversion, putting it back in the original orientation. Surprisingly, the two major inversions are not bordered by transposable elements.

**Minor Inversions** Minor rearrangements also occurred in other regions of the 70-kb plasmid. The gene encoding YopM represents a nice illustration of such rearrangements. Gene *yopM* itself shows some heterogeneity among different *Yersinia* isolates, probably because it encodes a protein with internal repetitions (Boland et al., 1998b). In addition, the orientation of *yopM* varies. The gene is oriented clockwise in *Y. enterocolitica* and anti-clockwise in *Y. pseudotuberculosis* and *Y. pestis*. The region upstream and downstream from *yopM* contains three long repeated sequences and putative open reading frames (ORFs; Reisner and Straley, 1992). Genetic recombinations between these repeats could explain the observed inversion of *yopM*.

**Localized Mutations** The *yadA* gene also underwent some modifications during evolution of the 70-kb plasmid. The *yadA* gene varies in the different serotypes of *Y. enterocolitica* resulting in proteins of different size. Most importantly, the *yadA* gene of *Y. pestis* has a single base pair deletion that results in a shift in the reading frame of the gene and a reduction of the half life of the mRNA resulting in the loss of the protein (Portnoy et al., 1984; Rosqvist et al., 1988; Skurnik and Wolf-Watz, 1989; Hu et al., 1998b).

The information available in databases also indicates that *ylpA* is a pseudogene in *Y. pestis* (Hu et al., 1998a; Perry et al., 1998).

YscM2, YomA, PprA, and ORF80 are not encoded by pCD1 (Hu et al., 1998b; Iriarte and Cornelis, 1999a). According to the information available in the databases, the gene encoding YomA is also missing in pIB1 from *Y. pseudotuberculosis* and only a partial ORF encoding 166 amino acids with 100% identity to the N-terminus of YomA protein is found in the pYVe227 plasmid of *Y. enterocolitica* serotype O:3 and a short ORF encoding the first 53 amino acids of YomA is found in the pCD1 plasmid of *Y. pestis* (accession number X13882; Skurnik and Wolf-Watz, 1989; Hu et al., 1998b; Iriarte et al., 2002).

**Duplication** The pYVe227 plasmid from *Y. enterocolitica* contains two copies of *yscM* (Stainier et al., 1997), a hypothetical player of the negative regulatory loop (Rimpiläinen et al., 1992). In contrast, there is only one copy of the homologue *lcrQ* in *Y. pseudotuberculosis* and *Y. pestis*. Thus, *yscM* was presumably duplicated after the *Y. pestis* and *Y. pseudotuberculosis* diverged (see below).

**Lack of the ARS Resistance Genes** As we mentioned before, the pYV plasmid of low virulence pathogenic *Y. enterocolitica* strains harbor a transposon encoding arsenite resistance (Neyt et al., 1997). This transposon is absent from pIB1 and pCD1, which is in agreement with the hypothesis of its recent acquisition by a pYV plasmid from *Y. enterocolitica* (see above).

**Conclusions** The three plasmids clearly have a common ancestor of the RepFII A type. The pYVe227 plasmid is probably closer to this ancestor than pIB1 and pCD1 because *yopH* and *sycH* are still associated, and *yadA* and *ylpA* are intact (China et al., 1990; Stainier et al., 1997; Iriarte and Cornelis, 1999a).

The fact that the pYVe227 plasmid contains the remains of a transfer operon suggests that, in its origin, the plasmid was conjugative. The *tra* genes that are still conserved such as *ylpA* homologous to *traT* and *nuc* could have evolved to fulfill another, still unknown function.

As it is generally the case for the large plasmids, stability of the 70-kb plasmid is assured by a partition system. However, unlike large plasmids (such as F, R1 and R438), the 70-kb plasmid does not have a system like *hok/sok*, *isrB* or *pnd*, which kills plasmid-free segregants (Nielsen et al., 1991; Gerdes et al., 1992). This observation is in perfect agreement with the fact that *Yersinia* easily gives pYV<sup>-</sup> segregants when plated at 37°C on a medium deprived of Ca<sup>2+</sup> (Biot and Cornelis, 1988).



### High-pathogenicity Island (Elisabeth Carniel)

**GENERAL CHARACTERISTICS OF THE HPI** One of the major differences between low-pathogenicity strains (which are not mouse-lethal and induce intestinal infections of moderate severity in humans), and high-pathogenicity strains (which are mouse-lethal and cause severe systemic infections in humans) of *Yersinia* is the presence of a “high-pathogenicity island” or “HPI” (Carniel et al., 1996), which can be considered as an iron-uptake island (Carniel, 2001). This designation comes from the original observation that the presence of this island is strictly correlated with the expression of a high-pathogenicity phenotype (De Almeida et al., 1993). The HPI is restricted to certain subgroups of the three pathogenic species of *Yersinia*: serotypes I and III (with a 9-kb truncation at the left-hand part) of *Y. pseudotuberculosis* (Rakin et al., 1995; Buchrieser et al., 1998a), biotype 1B of *Y. enterocolitica*, and all three biotypes of *Y. pestis* (De Almeida et al., 1993).

All criteria defining a pathogenicity island (Hacker et al., 1997) are fulfilled by the HPI: 1) it is a large chromosomal DNA fragment (36 kb in *Y. pseudotuberculosis* and *Y. pestis*, and 43 kb in *Y. enterocolitica*), 2) it carries genes essential for the expression of a high-pathogenicity phenotype, 3) it incorporates several repeated sequences (IS1328, IS1329, IS1222 and IS1400, or IS100) and a mobility gene (bacteriophage P4-like integrase gene), 4) it is bordered on one side by a *tRNA* locus (*asn tRNA*), 5) the G+C content

of the open reading frames composing the yersiniabactin locus (60%) is much higher than that of the core genome (46–50%), and 6) the island is mobile in some strains.

The HPI confers on the bacteria the ability to capture iron ions bound to eukaryotic molecules via a high-affinity, iron-chelating system (e.g., siderophore) called “yersiniabactin” (Heesemann, 1987), and subsequently to disseminate in mammalian hosts and to cause systemic infections. The siderophore yersiniabactin is a small molecule (482 Da), which belongs to a subgroup of phenolate siderophores and contains a phenolic group as well as one thiazolidine and two thiazoline rings (Haag et al., 1993; Drechsel et al., 1995; Chambers et al., 1996; Gehring et al., 1998a). Its structure is identical in *Y. enterocolitica* and *Y. pestis* (Perry et al., 1999), and closely resembles those of pyochelin and anguibactin produced by *Pseudomonas aeruginosa* and *Vibrio anguillarum*, respectively. The yersiniabactin molecule has an extremely high affinity for ferric iron ( $K_D = 4 \times 10^{-36}$ ; Gehring et al., 1998a; Perry et al., 1999). The biochemical structures and functions of different components of the machinery involved in the nonribosomal biosynthesis of yersiniabactin are currently under investigation (Suo et al., 1999; Suo et al., 2000; Suo et al., 2001; Keating et al., 2000a; Keating et al., 2000b; Ino and Murabayashi, 2001).

**GENETIC ORGANIZATION OF THE HPI** The HPI is composed of two functionally distinct parts (Fig. 36): the 30-kb right-hand highly conserved

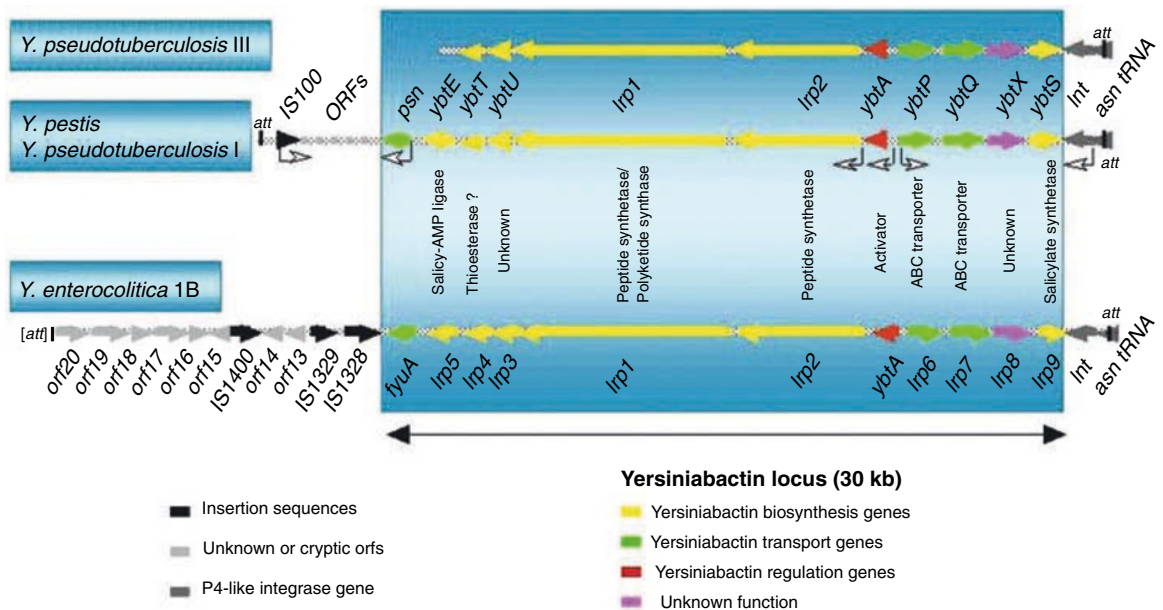


Fig. 36. Genetic organization of the HPIs of the three highly pathogenic species of *Yersinia*.

portion of the HPI, termed the “yersiniabactin locus,” and the left-hand part, which is variable in size and is much less conserved in the three highly pathogenic species.

**The Yersiniabactin Locus** This locus is composed of 11 genes, which can roughly be divided into three functional groups: yersiniabactin biosynthesis, transport into the bacterial cell, and regulation (Fig. 36).

The yersiniabactin biosynthesis genes are extremely conserved in highly pathogenic *Yersinia* species (> 98% nt identity; Buchrieser et al., 1998a; Gehring et al., 1998a; Rakin et al., 1999), and components of the yersiniabactin systems are interchangeable among the three species (Carniel et al., 1992; Perry et al., 1999). However, *Y. pseudotuberculosis* strains of serotype III lack the left-hand part of the yersiniabactin locus carrying the *ybtE* and *psn* genes. Formation of yersiniabactin occurs via a mixed polyketide synthase, nonribosomal peptide synthetase strategy that assembles the siderophore in modular fashion from salicylate (Guilvout et al., 1993; Gehring et al., 1998a; Gehring et al., 1998b; Suo et al., 1999; Keating et al., 2000a; Keating et al., 2000b). At least seven gene products are involved in yersiniabactin biosynthesis (Fig. 36): YbtU, YbtT, YbtE, YbtS, and the high-molecular weight proteins HMWP2 and HMWP1 (Geoffroy et al., 2000).

Three genes are involved in yersiniabactin-mediated internalization of iron: *psn*, *ybtP* and *ybtQ* (Fig. 36). Psn, a TonB-dependent outer-membrane protein, acts as a receptor for both yersiniabactin and the bacteriocin pesticin (Heesemann et al., 1993a; Fetherston and Perry, 1994; Rakin et al., 1994; Fetherston et al., 1995). YbtP and YbtQ are inner-membrane permeases required for translocation of iron into the bacterial cytosol (Gehring et al., 1998a; Fetherston et al., 1999). These two proteins are unique among the subfamily of ABC transporters of iron, since they both contain an amino-terminal membrane-spanning domain and a carboxy-terminal ATPase (Fetherston et al., 1999).

Several systems regulate expression of the yersiniabactin locus: 1) YbtA, a regulator of the AraC family of transcriptional regulators, activates expression from the *psn*, *irp2* and *ybtP* promoters, but represses expression of its own promoter (Fetherston et al., 1996); 2) the siderophore yersiniabactin may upregulate its own expression along with that of *psn* and *ybtPQXS* at the transcription level (Fetherston et al., 1996; Fetherston et al., 1999; Bearden et al., 1997; Peludat et al., 1998; Perry et al., 1999), probably by binding to the YbtA activator (Geoffroy et al., 2000); and 3) the Fur regulator represses transcription of the four promoter regions of the

yersiniabactin locus in the presence of iron (Guilvout et al., 1993; Rakin et al., 1994; Gehring et al., 1998a).

**The Right Border of the HPI** This region, well conserved among the three pathogenic *Yersinia* species, contains a gene homologous to that of the integrase of bacteriophage P4 and an asparagine tRNA (*asn tRNA*) locus (Carniel et al., 1996; Buchrieser et al., 1998a; Buchrieser et al., 1998b; Buchrieser et al., 1999; Bach et al., 1999; Hare et al., 1999; Rakin et al., 1999). The P4-like *int* gene may be potentially functional in *Y. pestis* and *Y. pseudotuberculosis*, but its homologue in *Y. enterocolitica* contains a premature stop codon that results in a truncated peptide (Bach et al., 1999; Rakin et al., 1999). The border of the HPI is formed by a 17-bp sequence homologous to the attachment site (*att*) of bacteriophage P4 and located at the 3' extremity of the *asn tRNA* locus.

**The Left-hand Region of the HPI** In contrast to the highly conserved 30-kb yersiniabactin locus, the left-hand portion of the island is polymorphic among various strains and species of *Yersinia* (Fig. 36) and can be subdivided into two main groups: *Y. enterocolitica* 1B on the one hand, and *Y. pestis* and *Y. pseudotuberculosis* I on the other hand (Rakin et al., 1995). This region is not yet defined in *Y. pseudotuberculosis* strains of serotype III.

In *Y. enterocolitica* 1B, the left-hand part, which extends 12.8 kb downstream of the *psn* gene, contains a cluster of four IS elements (IS1328, IS1329, a portion of IS1222 interrupted by IS1329, and IS1400), and seven other ORFs that have no significant homologies to any known gene (Rakin et al., 1995; Rakin et al., 1999; Rakin et al., 2000; Carniel et al., 1996). This region is not well conserved among various *Y. enterocolitica* 1B strains (Carniel et al., 1996). The left-hand extremity of the *Y. enterocolitica* HPI is delimited by a pseudo *att*-like site that represents a degenerate form of the 17 bp *att*-like site present at the other extremity of the island (Bach et al., 1999).

In *Y. pestis* and *Y. pseudotuberculosis* I, the left-hand portion of the HPI is approximately 5 kb long (Fig. 36). It carries one insertion sequence, designated “IS100” (Fetherston and Perry, 1994; Podladchikova et al., 1994; Prentice and Carniel, 1995) and several short ORFs homologous to phage genes (Buchrieser et al., 1999; Rakin et al., 1999). The left-hand part of the HPI terminates 248 and 516 bp downstream of the IS100 sequence in *Y. pseudotuberculosis* and *Y. pestis*, respectively (Buchrieser et al., 1998a; Buchrieser et al., 1999; Rakin et al., 1999). It has been suggested that the larger size of the region between the IS100 gene and the border of

Table 28. Effect of the mutation of various HPI-borne genes on the virulence of high-pathogenicity *Yersinia* in a mouse model of infection.

<i>Yersinia</i> species	Strain	Mutated gene	Route of infection	LD50		References
				parental strain	LD50 mutant	
<i>Y. pseudotuberculosis</i>	IP32790	<i>irp2</i>	IV	<10	$1.9 \times 10^3$	Carniel et al., 1992
	IP32790	<i>irp2</i>	SC	$3.5 \times 10^6$	$>2.1 \times 10^9$	Carniel et al., 1992
	IP32790	<i>irp2</i>	IG	$7.6 \times 10^7$	$2.7 \times 10^9$	Carniel et al., 1992
<i>Y. enterocolitica</i>	WA	<i>fyuA</i>	IV	$5 \times 10^2$	$>5 \times 10^6$	Rakin et al., 1994
<i>Y. pestis</i>	KIM5 ( $\Delta$ <i>psa</i> , <i>yopJ</i> )	<i>irp2</i>	SC	$1.3 \times 10^2$	$>1.3 \times 10^6$	Bearden et al., 1997
	KIM5 ( $\Delta$ <i>psa</i> , <i>yopJ</i> )	<i>psn</i>	SC	$1.3 \times 10^2$	$>2.9 \times 10^5$	Bearden et al., 1997
<i>Y. pestis</i>	KIM5 ( $\Delta$ <i>psa</i> , <i>yopJ</i> )	<i>ybtP</i>	SC	$1.1 \times 10^2$	$>7.6 \times 10^4$	Fetherston et al., 1999

Abbreviations: HPI, high pathogenicity island; IV, intravenously; SC, subcutaneously; and IG, intragastrically.

the HPI in *Y. pestis* is a scar of the ancient insertion and excision of an IS630-like element in this region (Hare et al., 1999). The left border of the island is determined by a perfectly conserved 17-bp sequence, repeated at the other extremity of the HPI (Buchrieser et al., 1998a; Buchrieser et al., 1999; Hare et al., 1999).

#### ROLE OF THE HPI IN *YERSINIA* PATHOGENESIS

Microbial pathogens must adapt to the iron-restricted milieu found in vivo. The successful establishment of disease depends on the ability of the invading organism to develop high-affinity iron transport systems. Numerous clinical reports have shown that *Y. enterocolitica* strains of biotypes 2 and 4, naturally deprived of the HPI-borne yersiniabactin system, are responsible for moderate intestinal symptoms, but can cause systemic infections if the patients are iron-overloaded (during thalassemia, hemochromatosis, etc.; Other putative or proven virulence factors, paragraph iron uptake systems). Similarly, in the mouse experimental model of infection, these strains do not kill mice at low doses unless iron or the siderophore Deferoxamine is provided exogenously (Robins-Browne and Prpic, 1985a). In contrast, *Y. enterocolitica* 1B strains, which carry the high-affinity iron-capture yersiniabactin system, are inherently lethal for laboratory animals at low doses. Therefore, although other as yet unidentified factors may participate in the high-pathogenicity phenotype, one of the major differences between low- and high-pathogenicity *Yersinia* lies in their ability to capture the iron molecules necessary for their systemic dissemination in the host via the yersiniabactin system.

Initially, indirect evidence for a role of the HPI in the expression of a high pathogenicity phenotype was obtained through the observation of a strict correlation between the presence of HPI-specific genes or products and the level of pathogenicity of a large number of natural *Yersinia* isolates (Harrison et al., 1980; Carniel et al.,

1987; Carniel et al., 1989; Heesemann, 1987; De Almeida et al., 1993; Heesemann et al., 1993a; Chambers and Sokol, 1994; Rakin et al., 1995). Definitive proofs for its role in *Yersinia* pathogenesis were acquired by mutagenizing various HPI-borne loci. Abolition of yersiniabactin production by uncharacterized Tn5 insertions in the chromosome of a *Y. enterocolitica* 1B resulted in an alteration of the pathogenicity of the mutants (Heesemann, 1987). Specific mutagenesis of various genes involved in yersiniabactin-mediated iron-uptake led to a reduction of virulence of the mutant for mice (Table 28). This decrease in virulence was much more important when the mutant was injected subcutaneously (SC) or intragastrically (IG) than when it was injected intravenously (IV). Similar results were recently obtained with a *Y. pseudotuberculosis* strain where the entire HPI was precisely excised from the chromosome (Lesic et al., 2002). The HPI thus seems to play a crucial role to allow the bacteria to disseminate from a peripheral portal of entry and to invade its host.

**MOBILITY OF THE HPI** The HPI inserts specifically into an *asn tRNA* locus present in three copies on the *Yersinia* chromosome (Buchrieser et al., 1998a). The *Y. pseudotuberculosis* HPI can insert into any of these three copies (Buchrieser et al., 1998a), while the HPI of *Y. enterocolitica* is inserted specifically into the *asnT* locus (Carniel et al., 1996; Rakin et al., 1999), and that of *Y. pestis* into another copy of this locus (Hare et al., 1999). The 3' end of each chromosomal *asn tRNA* locus contains a 17-bp sequence homologous to the *att* site of bacteriophage P4 (Buchrieser et al., 1998a; Rakin et al., 2001). It is likely that original integration of the HPI occurred by site-specific recombination between one of these *att*-like sites on the *Yersinia* chromosome and its homologue on an episome carrying the HPI. Integration of the HPI into the chromosome produces a duplication of the *att*-like site at each extremity of the island, while

excision of the HPI regenerates a unique *att*-like sequence at the junction site and restores an intact *asn tRNA* locus (Buchrieser et al., 1998a).

The *Yersinia* HPI also carries a gene homologous to the integrase gene (*int*) of phage P4. The presence of the *int* and *att* loci is necessary and sufficient for the integration of the HPI into the bacterial chromosome. Indeed, cloning of these two loci on a suicide plasmid engendered its integration into any of the chromosomal *att* sites (Rakin et al., 2001). These two loci are also involved in HPI excision from the chromosome.

In *Y. enterocolitica* 1B, where the *int* gene is naturally mutated and one of the two *att* sites is degenerate, no strain containing a precisely deleted island could ever be detected (De Almeida et al., 1993; Rakin et al., 1995; Bach et al., 1999), suggesting an essential role of these loci in HPI deletion.

This role was confirmed by demonstrating that mutagenesis of the integrase gene of *Y. pseudotuberculosis* abolishes HPI excision (Lesic et al., 2002). In this latter species, the frequency of spontaneous HPI excision from the chromosome is ca.  $10^{-4}$  for strain IP32637 (Buchrieser et al., 1998a), but may vary greatly depending on the strain (Lesic et al., in preparation). The fact that in individual colonies of the same isolate, the HPI could be found inserted into one or another *asn tRNA* locus suggests that this element has retained the ability to excise from and reinsert into another location on the *Y. pseudotuberculosis* chromosome (Buchrieser et al., 1998a).

The excision of the *Y. pestis* HPI is not precise but occurs as part of a much larger chromosomal deletion of 102 kb designated “*pgm* locus” (Fetherston et al., 1992), which encompasses most of the HPI and extends further rightward over a 68-kb region. Spontaneous excision of the *pgm* locus occurs at very high ( $2 \times 10^{-3}$ ) frequencies (Hare and McDonough, 1999) by homologous recombination between the two *IS100* flanking copies (Fetherston et al., 1992; Fetherston and Perry, 1994). The HPI of *Y. pestis* has the potential to virtually excise precisely from the chromosome since it possesses a copy of the *att* locus at each extremity, and an intact P4-like integrase gene (Buchrieser et al., 1998a; Hare et al., 1999), but its deletion is possibly masked by the high deletion frequency of the 102-kb *pgm* locus.

**PRESENCE OF THE HPI IN OTHER SPECIES OF ENTEROBACTERIA** Although the HPI was initially found and described in the genus *Yersinia*, it was subsequently identified in other members of the Enterobacteriaceae family such as *E. coli*, *Enterobacter cloacae*, various species of *Klebsiella* (*K. pneumoniae*, *K. rhinoscleromatis*, *K. ozaenae*, *K. planticola* and *K. oxytoca*), and of

*Citrobacter* (*C. diversus* and *C. koseri*; Schubert et al., 1998; Schubert et al., 2000b; Karch et al., 1999; Bach et al., 2000; Johnson and Stell, 2000; Xu et al., 2000; Clermont et al., 2001; Gophna et al., 2001; Johnson et al., 2001), and in non-I serotypes of *Salmonella enterica* (Oeschläger et al., 2002). The HPI is the only pathogenicity island identified until now in different bacterial genera.

The HPI of *E. coli* and *Yersinia* are highly conserved, as demonstrated by the sequences of portions of *irp2* and *fyuA* that are more than 98% identical in the two genera (Rakin et al., 1995; Schubert et al., 1998; Schubert et al., 1999b). The *E. coli* HPI is more closely related to the island of *Y. pestis* and *Y. pseudotuberculosis* than to that of *Y. enterocolitica* (Rakin et al., 1995). The overall organization of the HPI is also well conserved among the different HPI-positive enterobacteria, although minor differences at the extremities of the island may be noted (Bach et al., 2000). An intact P4-like *int* gene is commonly present and shares 94% shiga toxin-producing *E. coli* (STEC) to 99% uropathogenic *E. coli* (UPEC) identity with the *Y. pestis* homologue (Karch et al., 1999; Schubert et al., 1999b). Partial or total deletion of *int* has been reported in a few enterobacteria isolates (Karch et al., 1999; Bach et al., 2000; Gophna et al., 2001), suggesting a process of stabilization of the island on the chromosome of these bacteria. The left-hand boundary of the HPI is more variable among enterobacteria. Some *E. coli* strains that contain an *irp2* locus lack the *fyuA* gene, while some STEC isolates that do harbor the *fyuA* locus still do not synthesize the protein (Karch et al., 1999), indicating that *FyuA* may be dispensable under certain circumstances. In addition to *fyuA*, the *IS100* element and the *att* boundary of the HPI may also be missing in some *E. coli* (Schubert et al., 1998; Schubert et al., 1999a; Karch et al., 1999; Bach et al., 2000), a situation reminiscent of the HPI of *Y. pseudotuberculosis* III. With very few exceptions (Bach et al., 2000; Clermont et al., 2001), the enterobacteria HPI is integrated into the *asnT* locus, which contains a perfectly conserved *att*-like site.

In *E. coli*, the HPI is found in a wide variety of pathotypes: enteroaggregative (EAEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC)/STEC (serotypes O:26:H11/H- and O:128:H2/H-), UPEC, and septicemic and meningitis *E. coli* (Schubert et al., 1998; Karch et al., 1999; Clermont et al., 2001; Gophna et al., 2001). There is a strong association between the presence of the HPI and the severity of the infection. The HPI is more frequently associated with pathogenic strains than with saprophytic clinical isolates (Schubert et al., 1998). In the *E. coli*

reference (ECOR) collection, 93% of the strains belonging to the pathogenic groups B2 and D, versus less than 30% of the strains belonging to nonpathogenic groups A and B1 harbor the HPI (Johnson and Stell, 2000; Clermont et al., 2001; Johnson et al., 2001). Furthermore, carriage of the HPI is extremely frequent in *E. coli* strains causing severe forms of infection: 89% of serotype 078, and 100% of serotype 02 *E. coli* strains isolated from poultry with acute septicemia (Gophna et al., 2001), 70–100% of clinical strains isolated from urine or blood (Schubert et al., 1998; Schubert et al., 2000b; Gophna et al., 2001), and 100% of *E. coli* strains that caused neonatal meningitis in humans (Clermont et al., 2001; Gophna et al., 2001) possessed the HPI.

However, some HPI-positive *E. coli* were isolated as commensal microorganisms from asymptomatic carriers. The same holds true for *Citrobacter*, *Enterobacter* and *Klebsiella*, which may persist as saprophytic bacteria in healthy humans. These observations thus suggest that the HPI per se is not sufficient to cause severe infections in humans, and that additional bacterial and/or host factors may be required. In *Yersinia*, for instance, in the absence of the pYV virulence plasmid, the organisms cannot establish an infection, even if they harbor the HPI. In the case of usually commensal enterobacteria, it may be hypothesized that immune suppression may be the host factor that enables HPI-bearing organisms to disseminate in vivo and cause nosocomial infections, while HPI-negative organisms would remain in their initial niche. Therefore, as recently suggested (Hacker and Carniel, 2001), the presence of the HPI is a key factor in determining whether the bacterium will remain localized at the site of infection or will invade its host, but this capability may also be dependent on the genetic background of the HPI-bearing bacterium and on the host status.

### Invasin (Ralph Isberg)

**INVASIN WITHIN THE INTESTINE** Shortly after they are ingested, the enteropathogenic *Yersinia* establish a close relationship with host cells that is presumably maintained throughout the disease process. Within the intestine, the bacteria are internalized by M cells (Autenrieth and Firsching, 1996a; Marra and Isberg, 1997; Clark et al., 1998), which lay over the lymphoid follicle-associated epithelium (Neutra, 1999; Immunology and Physiopathology). After translocation across the M cell into these lymphoid follicles, there is no evidence for any further localization of the bacteria within host cells (Heesemann et al., 1993a).

At the very earliest stages of disease, the organism establishes contact with host cell  $\beta$ 1-

chain integrin receptors on the surface of M cells (Marra and Isberg, 1997; Clark et al., 1998). The primary protein responsible for this interaction is the bacterial invasin protein, which binds to at least five different integrin receptors ( $\alpha$ 3 $\beta$ 1,  $\alpha$ 4 $\beta$ 1,  $\alpha$ 5 $\beta$ 1,  $\alpha$ 6 $\beta$ 1 and  $\alpha$ v $\beta$ 1 (Isberg and Leong, 1990b) and is responsible for the ability of the organism to enter M cells in the intestine. Invasin mutants unable to bind to integrin receptors do not enter M cells and do not efficiently translocate into the lymphoid follicle (Pepe and Miller, 1993; Marra and Isberg, 1997).

**STRUCTURE OF INVASIN** Invasin is able to promote efficient uptake of bacteria into normally nonphagocytic cultured cell lines, reminiscent of what is observed with intestinal M cells. The *Y. pseudotuberculosis* invasin is a member of the bacterial intimin/invasin family, which consists of cell adhesion proteins found on the surface of a variety of enteropathogens (McGraw et al., 1999). The N-terminal 503-residues are highly conserved among family members and are important for surface localization of the protein (Leong et al., 1990). The divergent C-terminal region contains the determinants involved in receptor recognition and consists of a series of structural repeats that show little sequence homology to each other. At the C-terminus of the *Y. pseudotuberculosis* protein, four immunoglobulin-like (Ig-like) repeats are arrayed in tandem on the bacterial cell surface, followed by a C-type lectin-like domain (D5; CTLD) at the extreme C-terminus (Hamburger et al., 1999). The CTLD (D5) and the most upstream Ig domain (D4) form a large interface with each other, producing a superdomain that acts as a mammalian cell adhesion module.

The analysis of point mutations in cell adhesion superdomain reveals that two noncontiguous sites are involved in binding integrin receptors. The first region is localized in a disulfide loop and centered around the residue Asp911 (Leong et al., 1995). It has been proposed that this residue performs a similar role to the Asp1495 located in the arg-gly-asp sequence of fibronectin (Fn), which is critical for the binding of this protein to the integrin receptor  $\alpha$ 5 $\beta$ 1 (Leahy et al., 1996). The second region is centered around residue Asp811, although residue changes in this region have much weaker effects than seen with Asp911 (Saltman et al., 1996). Fibronectin also has a binding determinant not contiguous to arg-gly-asp, called the “synergy region,” which appears to play a role in supporting integrin binding (Aota et al., 1994). Thus, although there is no detectable sequence similarity between invasin and fibronectin, sequence determinants involved in receptor recognition appear to be similar.

**INVASIN-PROMOTED UPTAKE INTO CULTURED CELLS** Three important factors enhance invasin-mediated uptake: 1) high-affinity binding of integrin receptors by the D4-D5 superdomain (Tran Van Nhieu and Isberg, 1993); 2) the ability of invasin monomers to undergo homotypic interactions (Dersch and Isberg, 1999); and 3) an increase in the concentration of integrin receptors available to bind invasin (Tran Van Nhieu and Isberg, 1993; Dersch and Isberg, 1999). Mutations that lower the affinity of the protein for receptors, deletion of a region of invasin necessary for homotypic interaction, and depletion of integrin receptors from the host cell all severely depress bacterial uptake, causing extra-cellular adhesion of the bacteria.

#### HIGH-AFFINITY BINDING OF INTEGRIN RECEPTORS

The most striking difference between invasin and fibronectin binding is the significantly higher affinity of invasin-receptor binding. This property is both critical for the protein to promote uptake, as well as a central virulence determinant for the microorganism. Mutation of Asp911 to glutamate in invasin allows *Y. pseudotuberculosis* to adhere to cells, but the lowered affinity caused by the lesion prevents bacteria from entering M cells and colonizing Peyer's patches in the mouse intestine (Marra and Isberg, 1997).

#### THE ROLE OF INVASIN HOMOTYPIC INTERACTIONS

The monomeric cell adhesion domain of invasin is inefficient at promoting uptake, whereas dimerization of this superdomain is able to promote uptake. Deletion analysis of the *Y. pseudotuberculosis* invasin indicates that one of the immunoglobulin (Ig) domains (called "D2") promotes the homotypic interaction necessary for efficient uptake. The *Y. enterocolitica* invasin protein, which lacks D2, is much less efficient at promoting uptake than the *Y. pseudotuberculosis* protein (Dersch and Isberg, 2000). The rate of recruitment of tyrosine-phosphorylated proteins to the phagocytic cup is considerably enhanced by dimerization of invasin, which may be a reason for the importance of multimerization (P. Dersch, personal communication).

#### THE IMPORTANCE OF SUBSTRATE AND RECEPTOR DENSITY

By increasing the concentration of both invasin and integrin receptors, one can partially bypass the requirements for high receptor-substrate affinity and multimerization. Bacteria expressing high levels of invasin derivatives lacking D2 can be internalized at efficiencies approaching that of bacteria expressing similar levels of wildtype protein (Dersch and Isberg, 2000). Presumably, placing receptor-substrate contacts in sufficiently close proximity allows intracellular signals necessary for uptake to be

sent from the phagocytic cup. At lower invasin concentrations, such signals would require dimerization of the protein.

**SUMMARY** The physical properties of the invasin protein make it uniquely efficient at promoting uptake by engaging integrin receptors. Signaling events that lead to uptake of the bacteria as a consequence of integrin engagement are less well defined than the determinants of adhesivity. Presumably, integrin engagement leads to actin rearrangements that result in engulfment of the bacterium. Recent work indicates that signaling from the integrin via the small GTPase Rac1 is critical for sending information necessary for uptake. Future work will certainly involve determining the nature of the signals within the mammalian cell that leads to internalization.

#### Urease (Michel Simonet)

All *Yersinia* species, except *Y. pestis*, are able to use urease to hydrolyze urea to ammonia and carbon dioxide. Urease, a trimeric protein complex requiring nickel ions to be active (metalloenzyme), is exclusively located in the bacterial cytosol. De Koning-Ward and Robins-Browne (1997) showed that *Yersinia* urease is a novel type of bacterial urease because 1) it is maximally active at acidic pH and 2) it has a high affinity for urea, with an extremely low  $K_m$  value.

The genes required for urease biosynthesis have been characterized in the three pathogenic species (GenBank accession numbers {L24101} for *Y. enterocolitica*, {U40842} for *Y. pseudotuberculosis* and {AF095636} for *Y. pestis*). Three adjacent genes, *ureA*, *ureB* and *ureC*, encode the structural subunits, UreA (11 kDa), UreB (16–18 kDa) and UreC (61 kDa), which together form an inactive form of the enzyme (urease apoenzyme). The incorporation of nickel ions into the enzymatic catalytic site, which is located in UreC, requires at least four additional genes (*ureE*, *ureF*, *ureG* and *ureD*) that are contiguous to the chromosomal structural genes. As expected, the gene cluster is highly homologous between pathogenic *Yersinia* species (especially for *Y. pseudotuberculosis* and *Y. pestis*), and the structural genes are more highly conserved than the accessory genes (De Koning-Ward et al., 1994; Sebbane et al., 2001). Several genetic features suggest that *ure* genes are arranged in an operon-like manner, but it is not known whether they form a single (*ureABCEFGD*) or two polycistronic units (*ureABC* and *ureEFGD*). *Yersinia pestis* produces an inactive form of urease; this is due to the presence of one additional G residue in a poly G stretch which introduces a premature stop codon in *ureD* and leads to the truncation

of the accessory protein, UreD (Sebbane et al., 2001).

Unlike in most bacterial species, the expression of the *Yersinia* urease gene cluster is not induced by nitrogen limitation or by urea (De Koning-Ward and Robins-Browne, 1997). This is consistent with the absence of a binding site for the alternative sigma factor,  $\sigma^{54}$ , which governs transcription of nitrogen-regulated genes, within the *ureA* promoter region. Additionally, unlike some bacterial urease gene clusters, no urea-inducible activator gene (*ureR*) has been identified upstream of *ureA* in *Yersinia* species (Mobley et al., 1995). *Yersinia*'s ureolytic activity is growth phase-dependent and is optimal during the stationary phase, at both 28 and 37°C; the regulatory gene involved has not been identified yet, but it is not the gene encoding the RpoS  $\sigma$  factor.

Nonpathogenic and pathogenic (except *Y. pestis*) yersiniae are soil- and water-dwelling microorganisms. Urea is present in these environments and represents the main nitrogenous waste product of mammals; its degradation by urease provides bacteria with ammonium ions, an easily assimilable nitrogen source (Mobley and Hausinger, 1989). Therefore, the primary use of urease probably is to allow *Yersinia* spp. to live as saprophytes. However, it has been suggested that urease may also contribute to the pathogenesis of enteropathogenic species *Y. enterocolitica* and *Y. pseudotuberculosis* by protecting the microorganisms during their passage through the stomach. This is supported by the fact that in vitro, the enzyme is active at acidic pH and in the presence of low concentrations of urea, such as those found in the gastric juice. Isogenic urease-deficient mutants from both species were significantly disabled in acid stress conditions in vitro when compared to the wildtype strains, thus confirming this hypothesis (De Koning-Ward and Robins-Browne, 1995; Young et al., 1996; Riot et al., 1997). Furthermore, in an experimental model of murine oral infection, it was found that less urease-negative *Y. enterocolitica* mutants were recovered from the ileum of mice 90 min after the ingestion of contaminated drinking water or by gavage than wildtype *Y. enterocolitica* (De Koning-Ward and Robins-Browne, 1995); however, this difference was not observed with the corresponding *Y. pseudotuberculosis* mutant (Riot et al., 1997).

Enteric infection by *Y. pseudotuberculosis* and particularly *Y. enterocolitica* is sometimes complicated by reactive arthritis, especially when it occurs in patients positive for HLA-B27 (Clinical manifestations of *Y. enterocolitica* and Immunology). During a systematic search for bacterial arthritogenic factors, Mertz et al.

(1991) isolated a 19-kDa cationic protein from a *Y. enterocolitica* extract, which triggered joint inflammation when injected intra-articularly into presensitized rats. Probst et al. (1993) and Skurnik et al. (1993) concomitantly identified the 19-kDa protein as the UreB structural subunit of urease. Interestingly, this molecule was recognized as an immunodominant antigen by T lymphocytes from synovial fluids from humans with *Yersinia*-triggered reactive arthritis (Probst et al., 1993; Mertz et al., 1998; Appel et al., 1999); the 19-kDa protein also induced a significant humoral immune response in these patients, and the detection of serum antibodies has been shown to be useful for the diagnosis of reactive arthritis. These results suggest that UreB plays a role in the induction of arthritis. However, by using the experimental model of arthritis in the rat, Gripenberg-Lerche et al. (2000) found that the incidence of arthritis was almost the same when animals were inoculated intravenously with a wildtype *Y. enterocolitica* strain or with an isogenic *ureB*-deficient mutant; nevertheless, the authors noted that the onset of the illness was slightly delayed (but not significantly) and that the severity of joint inflammation was somewhat milder in rats infected with the mutant.

### Lipopolysaccharide (Mikael Skurnik)

**O-SEROTYPES OF YERSINIA** Serotyping as a means of classifying *Salmonella* isolates was started as early as 1926 (White, 1926; Kauffmann, 1966) and is known as the Kauffmann-White scheme. The primary antigens in the scheme are the cell surface lipopolysaccharides (O-antigens) and the flagellin proteins (H-antigens). The O-antigen epitopes are determined by the type, arrangement, and condition of sugar residues in the repeated O-units. O-serotyping was applied to *Y. pseudotuberculosis* (5 serotypes) by Thal in 1954 (Thal, 1954) and to *Y. enterocolitica* (8 serotypes) by Winblad in 1967 (Winblad, 1973).

Since then, a substantial number of new serotypes have been added to both serotyping schemes: *Y. pseudotuberculosis* is now subdivided into 21 serotypes (Link 1: *Y. pseudotuberculosis* serotypes).

Serologically, *Y. pestis* strains are very homogeneous because their lipopolysaccharide (LPS) is rough, i.e., is lacking O-antigen (Brubaker, 1991). *Yersinia pestis* is apparently a very recently evolved clone of *Y. pseudotuberculosis* O:1b (Achtman et al., 1999; Skurnik et al., 2000) carrying a cryptic O-antigen gene cluster that is almost identical to that of *Y. pseudotuberculosis* O:1b (Skurnik et al., 2000).

*Yersinia enterocolitica* strains (including *Y. enterocolitica*-like species) are subdivided into

Table 29. Characteristics of *Y. pseudotuberculosis* serotypes.

O-serotype	O-factors	Presence of the virulence plasmid	<i>ddh</i> -genes	DDH
O:1a	2, 3 <sup>a</sup> , 23	pYV	<i>ddhDABC</i>	Par
O:1b	2, 4 <sup>a</sup> , 23	pYV	<i>ddhDABC</i>	Par
O:1c	2, 3, 17, 24 <sup>a</sup>	pYV	<i>ddhDABC</i>	UN
O:2a	5, 6 <sup>a</sup> , 16	pYV	<i>ddhDABC</i>	Abe
O:2b	5, 7 <sup>a</sup> , 16, 17	pYV	<i>ddhDABC</i>	Abe
O:2c	5, 7, 11, 18 <sup>a</sup>	pYV	<i>ddhDABC</i>	Abe
O:3	8 <sup>a</sup> , 15	pYV	<i>ddhDABC</i>	Par
O:4a	9, 11 <sup>a</sup>	pYV	<i>ddhDABC</i>	Tyv
O:4b	9, 12 <sup>a</sup>	pYV	<i>ddhDABC</i>	Tyv
O:5a	10, 14 <sup>a</sup>	pYV	<i>ddhDABC</i>	Asc
O:5b	10, 15 <sup>a</sup>	pYV	<i>ddhDA</i>	6d-Alt <sup>f</sup>
O:6	13 <sup>a</sup> , 19, 26	pYV	<i>ddhDABC</i>	Col
O:7	13, 19 <sup>a</sup>	UN	UN	Col
O:8	11, 20 <sup>a</sup>	pYV	<i>ddhDABC</i>	UN
O:9	10, 25 <sup>a</sup>	UN	UN	UN
O:10	26 <sup>a</sup>	pYV	UN	UN
O:11	4, 15, 27 <sup>a</sup>	UN	<i>ddhDA</i>	UN
O:12	18, 27, 28 <sup>a</sup>	UN	<i>ddhDABC</i>	UN
O:13	28, 29 <sup>a</sup>	UN	<i>ddhDABC</i>	UN
O:14	13, 30 <sup>a</sup>	UN	<i>ddhDA</i>	UN
O:15	UN	UN	<i>ddhDABC</i>	UN

Abbreviations: *ddh*, dideoxyhexose gene; Par, paratose; Abe, abequose; Tyv, tyvelose; Asc, ascarylose; Col, colitose; 6d-Alt<sup>f</sup>: 6-deoxy-L-altrofuranoise; DDH, dideoxyhexose sugar; and UN, unknown.

<sup>a</sup>Serotype-specific factor.

From Tsubokura and Aleksic (1995).

more than 70 serotypes (Link 2: Serotypes of *Y. enterocolitica* and related species).

While most *Y. pseudotuberculosis* serotypes appear to be associated with pathogenicity (Nagano et al., 1997b), this is true only for a few of *Y. enterocolitica* serotypes and biotypes.

**CHEMICAL STRUCTURES** The chemical structures of a number of *Yersinia* LPS molecules or different parts of the molecules have been determined. In most cases, the structure of the repeating O-unit has been resolved and only in a few cases the lipid A and core structures are known (Table 31). A peculiarity of *Y. enterocolitica* is the presence of a branching outer-core oligosaccharide in some *Y. enterocolitica* serotypes (e.g., O:3) expressing homopolymeric O-antigen (Skurnik et al., 1995). Only limited attention has been paid to the variability of the chemical structure of the LPS molecule with regard to different growth conditions, e.g., growth temperature, pH, ionic strength, etc. (Link 6. Regulation of LPS biosynthesis).

**LPS BIOSYNTHESIS** Gram-negative bacteria, like the members of the genus *Yersinia*, possess an outer membrane (OM). Most of the outer leaflet of the OM is occupied by LPS, which is com-

posed of two biosynthetic entities: 1) lipid A-core oligosaccharide, and attached to it, 2) O-specific polysaccharide also known as O-antigen. This division has relevance for the understanding of the biology and genetics of LPS; therefore, biosynthesis of these two LPS entities will be briefly discussed below (Link 3: LPS biosynthesis). Purified LPS is traditionally visualized by silver staining after SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Fig. 39), which reveals that bacteria express a heterogeneous population of LPS molecules with an Mr range of ~2000 to 50,000. This is because some LPS molecules do not carry any O-specific polysaccharide chains at all (Mr ~2000), while others do have a large variation in the lengths of the individual polysaccharide chains (Mr > 2000–50,000).

#### ENTEROBACTERIAL COMMON ANTIGEN AND LPS

Enterobacterial common antigen (ECA) is a polysaccharide with a trisaccharide repeat unit expressed by all Enterobacteriaceae including *Yersinia* (Kuhn et al., 1988; Rick and Silver, 1996; Radziejewska-Lebrecht et al., 1998). ECA can be in three different structural forms: 1) linked to L-glycerophosphatide, 2) linked to LPS, or 3) as a cyclic structure (Rick and Silver, 1996). When linked to LPS, ECA is ligated specifically



Table 30. Some characteristics of the serotypes of *Y. enterocolitica* and related species.

Serotype	Species	Presence of the virulence plasmid	Presence of IVS in 23S rDNA	Presence of O:3 type LPS outer core
O:1	<i>Y. enterocolitica</i>	pYV	IVS	OC
O:1,2,3	<i>Y. enterocolitica</i>	pYV	n.t.	—
O:1,3	<i>Y. enterocolitica</i>	pYV	n.t.	n.t.
O:2	<i>Y. enterocolitica</i>	pYV	IVS	OC
O:3	<i>Y. enterocolitica</i>	pYV	IVS	OC
O:3	<i>Y. kristensenii</i>	—	n.t.	n.t.
O:4	<i>Y. enterocolitica</i>	pYV	n.t.	—
O:4,32	<i>Y. enterocolitica</i>	pYV	IVS	—
O:4,33	<i>Y. enterocolitica</i>	—	n.t.	n.t.
O:5	<i>Y. enterocolitica</i>	—	n.t.	n.t.
O:5,27	<i>Y. enterocolitica</i>	pYV	IVS	OC
O:6,30	<i>Y. enterocolitica</i>	—	—	—
O:6,31	<i>Y. enterocolitica</i>	—	—	OC
O:7,8	<i>Y. enterocolitica</i>	—	—	—
O:8	<i>Y. enterocolitica</i>	pYV	IVS/—	—
O:9	<i>Y. enterocolitica</i>	pYV	IVS	OC
O:10	<i>Y. enterocolitica</i>	—	—	—
O:11,23	<i>Y. enterocolitica</i>	—	n.t.	n.t.
O:12,25	<i>Y. kristensenii</i>	—	—	—
O:13,7	<i>Y. enterocolitica</i>	pYV	n.t.	—
O:13,18	<i>Y. enterocolitica</i>	pYV	IVS	—
O:13a,13b	<i>Y. enterocolitica</i>	pYV	IVS	—
O:14	<i>Y. enterocolitica</i>	—	n.t.	—
O:16	<i>Y. kristensenii</i>	—	—	—
O:16	<i>Y. frederiksenii</i>	—	—	—
O:16,21	<i>Y. intermedia</i>	—	—	—
O:20	<i>Y. enterocolitica</i>	pYV	IVS	—
O:21	<i>Y. enterocolitica</i>	pYV	IVS	—
O:25	<i>Y. enterocolitica</i>	—	—	—
O:25,26,44	<i>Y. enterocolitica</i>	—	n.t.	OC
O:26,44	<i>Y. enterocolitica</i>	—	n.t.	—
O:28,50	<i>Y. enterocolitica</i>	—	—	—
O:34	<i>Y. enterocolitica</i>	pYV	IVS	—
O:35	<i>Y. frederiksenii</i>	—	n.t.	—
O:35,36	<i>Y. enterocolitica</i>	—	n.t.	—
O:35,52	<i>Y. enterocolitica</i>	—	—	—
O:41(27),42	<i>Y. enterocolitica</i>	—	—	—
O:41(27)43	<i>Y. enterocolitica</i>	—	n.t.	OC
O:41(27)K1	<i>Y. enterocolitica</i>	—	n.t.	—
O:41,43	<i>Y. enterocolitica</i>	—	n.t.	OC
O:48	<i>Y. frederiksenii</i>	—	—	—
O:50	<i>Y. enterocolitica</i>	—	—	OC
O:52,54	<i>Y. intermedia</i>	—	—	OC
O:58,16	<i>Y. frederiksenii</i>	—	n.t.	—
O:58,16	<i>Y. bercovieri</i>	—	IVS/—	—
O:59(20,35,7)	<i>Y. mollaretii</i>	—	—	—

Symbols and abbreviations: —, absent; pYV, ~70kb virulence plasmid of *Yersinia*;

IVS, a ~100-bp intervening sequence in the 23S rRNA gene; IVS/—, IVS is not present in all rRNA operons; OC, outer core oligosaccharide; and n.t., not tested.

From Skurnik (1985), Skurnik and Toivanen (1991), and Skurnik et al. (1995).

to those lipid A-core molecules that do not carry O-antigen. ECA biosynthesis on undecaprenyl phosphate (Und-P) takes place using similar reactions as described above for heteropolymeric O-antigen, and ligation to LPS is WaaL (O-polysaccharide ligase)-dependent (Rick and Silver, 1996). LPS-linked ECA is immunogenic

and antibodies against ECA are formed during infection.

**GENETICS OF YERSINIA LPS BIOSYNTHESIS** The genetic work with *Yersinia* has been performed mainly on the O-antigen gene clusters and the outer-core gene cluster of *Y. enterocolitica* O:3.

Table 31. Chemical structures of the lipopolysaccharides of the genus *Yersinia*.

Species, serotype	Structure. Unless otherwise indicated the structure of the O-unit is given.	References
<i>Y. enterocolitica</i> O:3, O:9	<p>Outer core<sup>1</sup></p> <p>Glc β1,6 GalNAc α1,6 Gal-(α1→4)-GalNAc-(α1→3)-Fuc2NAc-(β1→3)-LD-Hep-(α1→3)-LD-Hep-(α1→5)-KDO→lipid A β1,3 Glc</p> <p>Core</p> <p>DD-Hep α1,7 LD-Hep α1,7 β1,2 Glc</p> <p>Inner core</p> <p>DD-Hep α1,7 LD-Hep α1,7 β1,4 Glc</p> <p>Lipid A</p>	(Müller-Loennies et al., 1999; Ovodov et al., 1992; Radziejewska-Lebrecht et al., 1994; Shashkov et al., 1995)
<i>Y. enterocolitica</i> O:8	<p>Core</p> <p>DD-Hep α1,7 LD-Hep α1,7 β1,2 Glc</p> <p>(Non-stoichiometric) GlcNAc-(β1→3)-LD-Hep-(α1→3)-LD-Hep-(α1→5)-KDO-(α2→6)-GlcN β1,4 Glc KDO GlcN-(1←)-P</p> <p>Lipid A</p>	(Oertelt et al., 2001)
<i>Y. pseudotuberculosis</i>	<p>Core</p> <p>Hep α1,7 Hep α1,7 β1,2 Glc (Non-stoichiometric)</p> <p>Glc-(β1→4)-Glc-(1→?) -Glc-(α1→3)-Hep-(α1→3)-Hep-(1→?) -KDO→ lipid A β1,7 GlcNAc Gal β1,3 Glc</p>	(Ovodov et al., 1992)
<i>Y. aldovae</i> (Fuc3NIR <sup>2</sup> )	<p>→6)-GlcNAc-(α1→4)-GalNAc-(β1→3)-GlcNAc-(β1→2)-Glc-(β1→2)-Fuc3NR-(α1→</p>	(Zubkov et al., 1991)
<i>Y. bercovieri</i> O:10 (YerA <sup>3</sup> )	<p>→3)-D-Rha-(α1→3)-D-Rha-(α1→</p> <p>YerA</p>	(Gorshkova et al., 1994)
<i>Y. enterocolitica</i> O:1,2a,3 (6d-Alt <sup>4</sup> )	<p>→2)-6d-Alt-(β1→2)-6d-Alt-(β1→3)-6d-Alt-(β1→</p>	(Gorshkova et al., 1985; Hoffman et al., 1980)
<i>Y. enterocolitica</i> O:2a,2b,3	<p>→2)-6d-Alt-(β1→2)-6d-Alt(β1→3)-6d-Alt-(β1→</p> <p>↑3 OAc</p> <p>→2)-6d-Alt-(β1→</p>	(Gorshkova et al., 1985; Hoffman et al., 1980)
<i>Y. enterocolitica</i> O:3	<p>→2)-6d-Alt-(β1→</p>	(Gorshkova et al., 1985; Hoffman et al., 1980)

<i>Y. enterocolitica</i> O:4,32 (YerB <sup>5</sup> )	(1'AcO)YerB $\alpha\downarrow,1,4$ →3)-GalNAc-( $\alpha 1 \rightarrow 3$ )-GalNAc-( $\beta 1 \rightarrow$ →3)-L-Rha-( $\beta 1 \rightarrow 3$ )-L-Rha-( $\alpha 1 \rightarrow$ $\beta \uparrow 2,2$ Xlu <sup>f</sup>	(Gorshkova et al., 1987; Zubkov et al., 1989)
<i>Y. enterocolitica</i> O:5,27 (Xlu <sup>f6</sup> )	or →3)-L-Rha-( $\alpha 1 \rightarrow 3$ )-L-Rha-( $\alpha 1 \rightarrow 3$ )-L-Rha-( $\beta 1 \rightarrow$ $\beta \uparrow 2,2$ Xlu <sup>f</sup>	(Gorshkova et al., 1986; Perry and MacLean, 1987)
<i>Y. enterocolitica</i> O:6,31 (6d-Gul <sup>7</sup> )	→2)-D-Gal-( $\alpha 1 \rightarrow 3$ )-6d-Gul-( $\beta 1 \rightarrow$	(Kalmykova et al., 1988)
<i>Y. enterocolitica</i> O:8	→4)-D-Man-(1→3)-D-Gal-(1→3)-D-GalNAc-(1→ $\uparrow 1,3$ $\uparrow 1,2$ 6d-Gul L-Fuc	(Tomshich et al., 1987)
<i>Y. enterocolitica</i> O:7,8 and O:19,8	6d-Gul $\downarrow 1,4$ →2)-D-Man-(1→3)-D-Gal-(1→3)-D-GalNAc-(1→ $\uparrow 1,3$ $\uparrow 1,2$ 6d-Gul L-Fuc	(Ovodov et al., 1992; Tomshich et al., 1987)
<i>Y. enterocolitica</i> O:9 (perosamine <sup>8</sup> )	→2)-4,6-dd-4-formamido- $\alpha$ -D-man-(1→	(Caroff et al., 1984)
<i>Y. enterocolitica</i> O:10 (L-Xlu <sup>9</sup> )	→3)-D-Rha-( $\alpha 1 \rightarrow 3$ )-D-Rha-( $\alpha 1 \rightarrow$ $\alpha \uparrow 1,3$ L-Xlu	(Gorshkova et al., 1995)
<i>Y. enterocolitica</i> O:11,23 and O:11,24 <sup>10</sup>	→3)-L-QuiNAc-(1 $\alpha$ →4)-D-GalNAc-( $\alpha 1 \rightarrow 3$ )-L-QuiNAc-( $\beta 1 \rightarrow 3$ )-D-GlcNAc-( $\alpha 1 \rightarrow$	(Marsden et al., 1994)
<i>Y. enterocolitica</i> O:28	→3)-L-Rhap-( $\beta 1 \rightarrow 3$ )-D-GlcNAc-( $\alpha 1 \rightarrow 3$ )-L-Rhap-( $\alpha 1 \rightarrow 3$ )-L-Rhap-( $\alpha 1 \rightarrow$ $\alpha \uparrow 1,2$ L-Rhap-( $\alpha 1 \rightarrow 4$ )-D-GalpNAcA	(Perry and MacLean, 2000)
<i>Y. frederiksenii</i> O:16,29	→3)-D-Rha-( $\alpha 1 \rightarrow 3$ )-D-Rha-( $\alpha 1 \rightarrow 2$ )-D-Rha-( $\beta 1 \rightarrow$ $\beta \uparrow 1,2$ YerA (33%) $\uparrow 6$ AcO	(Gorshkova et al., 1989)
<i>Y. intermedia</i> strain 680 (fructane <sup>11</sup> )	→1)-Fru <sup>f</sup> -( $\beta 2 \rightarrow 1$ )-Fru-( $\alpha 1 \rightarrow$ $\uparrow 6$ AcO	(Gorshkova et al., 1987)
<i>Y. intermedia</i> O:4,33	YerB $\alpha\downarrow,1,4$ →3)-GalNAc-( $\alpha 1 \rightarrow 3$ )-GalNAc-( $\beta 1 \rightarrow$	(Zubkov et al., 1988)
<i>Y. kristensenii</i> O:25,35	→6)-Glc-( $\alpha 1 \rightarrow 4$ )-FucNAc-( $\beta 1 \rightarrow 3$ )-GlcNAc-(1→2)-Gro-( $\beta 1$ -P→ $\alpha \uparrow 1,3$ GalNAc $\alpha \uparrow 1,6$ Glc	(Gorshkova et al., 1993)

Table 31. *Continued*

Species, serotype	Structure. Unless otherwise indicated the structure of the O-unit is given.	References
<i>Y. kristensenii</i> O:12,25 hexaosylglycerol phosphate structures <sup>12</sup>	→6)-Glc-(α1→4)-FucNAc-(β1→3)-GlcNAc-(1→2)-Gro-(β1-P→ α↓1,3 GalNAc α↓1,6 GlcNAc Glc	(L'vov et al., 1992)
<i>Y. kristensenii</i> O:12, 26	→6)-Glc-(β1→6)-GalNAc-(α1→3)-FucNAc-(α1→3)-GlcNAc-(β1→2) Gro-(1-P→ α↓1,2 Glc	(L'vov et al., 1990)
<i>Y. pseudotuberculosis</i> O:1a <sup>13</sup>	Par α↓1,3 6d-manno-Hep β↓1,4 →3)-Gal-(α1→3)-GlcNAc-(β1→ Par ↓1,3	(Komandrova et al., 1984; Samuelsson et al., 1974)
<i>Y. pseudotuberculosis</i> O:1b	→2)-Man-(1→4)-Man-(1→3)-Fuc-(1→3)-GlcNAc-(1→ Abe ↓1,3	(Tomshich et al., 1976)
<i>Y. pseudotuberculosis</i> O:2a	6d-manno-Hep ↓1,4 →3)-Gal-(1→ Abe ↓1,3	(Samuelsson et al., 1974)
<i>Y. pseudotuberculosis</i> O:2b	→2)-Man-(1→3)-α-Fuc-(1→ Abe α↓1,6	(Samuelsson et al., 1974)
<i>Y. pseudotuberculosis</i> O:2c	→3)-Man-(α1→2)-Man-(β1→2)-Man-(α1→3)-GalNAc-(α1→ Par β↓1,4	(Gorshkova et al., 1991)
<i>Y. pseudotuberculosis</i> O:3	→3)-Fuc-(α1→3)-GalNAc-(α1→2)-Man-(α1→ Tyv α↓1,6	(Gorshkova et al., 1980; Isakov et al., 1983; Samuelsson et al., 1974)
<i>Y. pseudotuberculosis</i> O:4a	→3)-Man-(β1→3)-Man-(α1→2)-Man-(α1→3)-GalNAc-(α1→ Tyv α↓1,3	(Gorshkova et al., 1983; Samuelsson et al., 1974)
<i>Y. pseudotuberculosis</i> O:4b	6d-manno-Hep ↓1,4 →3)-Gal-(1→	(Samuelsson et al., 1974)

<i>Y. pseudotuberculosis</i> O:5a	$\begin{array}{l} \text{Asc} \\ \downarrow 1,3 \\ \rightarrow 2)\text{-Fuc-(1}\rightarrow 3)\text{-Man-(1}\rightarrow 4)\text{-Fuc-(1}\rightarrow 3)\text{-GalNAc-(1}\rightarrow \\ 6\text{d-Altf} \\ \downarrow 1,3 \\ \rightarrow 2)\text{-Fuc-(1}\rightarrow 3)\text{-Man-(1}\rightarrow 4)\text{-Fuc-(1}\rightarrow 3)\text{-GalNAc-(1}\rightarrow \\ \rightarrow 3)\text{-GlcNAc-(}\beta 1\rightarrow 6)\text{-GalNAc-(}\alpha 1\rightarrow 3)\text{-GalNAc-(}\beta 1\rightarrow \\ \beta 1,3 \\ \text{Col-(}\alpha 1\rightarrow 2)\text{-YerA} \end{array}$	(Gorshkova et al., 1983; Samuelsson et al., 1974)
<i>Y. pseudotuberculosis</i> O:5b	$\begin{array}{l} \rightarrow 2)\text{-Fuc-(1}\rightarrow 3)\text{-Man-(1}\rightarrow 4)\text{-Fuc-(1}\rightarrow 3)\text{-GalNAc-(1}\rightarrow \\ \rightarrow 3)\text{-GlcNAc-(}\beta 1\rightarrow 6)\text{-GalNAc-(}\alpha 1\rightarrow 3)\text{-GalNAc-(}\beta 1\rightarrow \\ \beta 1,3 \\ \text{Col-(}\alpha 1\rightarrow 2)\text{-YerA} \end{array}$	(Korchagina et al., 1982; Samuelsson et al., 1974)
<i>Y. pseudotuberculosis</i> O:6	$\begin{array}{l} \rightarrow 6)\text{-Glc-(}\beta 1\rightarrow 3)\text{-GalNAc-(}\alpha 1\rightarrow 3)\text{-GalNAc-(}\beta 1\rightarrow \\ \alpha 1,2 \\ \text{Col} \\ \rightarrow 3)\text{-L-Rha-(}\alpha 1\rightarrow 3)\text{-L-Rha-(}\beta 1\rightarrow 3)\text{-L-Rha-(}\alpha 1\rightarrow \\ \rightarrow 3)\text{-FucAm-(}\alpha 1\rightarrow 3)\text{-GlcNAc-(}\alpha 1\rightarrow 8)\text{-L-Sug-(}\alpha 2\rightarrow \\ \beta 1,4 \\ \text{GlcNAc} \end{array}$	(Gorshkova et al., 1983; Gorshkova et al., 1976)
<i>Y. pseudotuberculosis</i> O:7	$\begin{array}{l} \rightarrow 6)\text{-Glc-(}\beta 1\rightarrow 3)\text{-GalNAc-(}\alpha 1\rightarrow 3)\text{-GalNAc-(}\beta 1\rightarrow \\ \alpha 1,2 \\ \text{Col} \\ \rightarrow 3)\text{-L-Rha-(}\alpha 1\rightarrow 3)\text{-L-Rha-(}\beta 1\rightarrow 3)\text{-L-Rha-(}\alpha 1\rightarrow \\ \rightarrow 3)\text{-FucAm-(}\alpha 1\rightarrow 3)\text{-GlcNAc-(}\alpha 1\rightarrow 8)\text{-L-Sug-(}\alpha 2\rightarrow \\ \beta 1,4 \\ \text{GlcNAc} \end{array}$	(Kotandrova et al., 1989)
<i>Y. rohdei</i>	$\begin{array}{l} \rightarrow 3)\text{-L-Rha-(}\alpha 1\rightarrow 3)\text{-L-Rha-(}\beta 1\rightarrow 3)\text{-L-Rha-(}\alpha 1\rightarrow \\ \rightarrow 3)\text{-FucAm-(}\alpha 1\rightarrow 3)\text{-GlcNAc-(}\alpha 1\rightarrow 8)\text{-L-Sug-(}\alpha 2\rightarrow \\ \beta 1,4 \\ \text{GlcNAc} \end{array}$	(Zubkov et al., 1993)
<i>Y. ruckerii</i> O:1 <sup>14</sup>	$\begin{array}{l} \rightarrow 3)\text{-L-Rha-(}\alpha 1\rightarrow 3)\text{-L-Rha-(}\beta 1\rightarrow 3)\text{-L-Rha-(}\alpha 1\rightarrow \\ \rightarrow 3)\text{-FucAm-(}\alpha 1\rightarrow 3)\text{-GlcNAc-(}\alpha 1\rightarrow 8)\text{-L-Sug-(}\alpha 2\rightarrow \\ \beta 1,4 \\ \text{GlcNAc} \end{array}$	(Beynon et al., 1994)

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Table 31. Footnote Continued

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**Genetics of the Lipid A and Core Biosynthesis**  
 No genetic experiments have been reported on the lipid A and (inner) core biosynthesis genes of *Yersinia*. The basic genetic setup most likely resembles that of *E. coli* and *Salmonella*, however, very likely there will be differences in the regulation of the biosynthetic activities under different environmental conditions. The ongoing genomic sequencing work (*Y. pseudotuberculosis* O:1b and *Y. enterocolitica* O:8) will help to elucidate these issues.

**The *Yersinia* Heteropolymeric O-antigen Gene Clusters are Between *hemH* and *gsk***  
 In *Yersinia*, all the studied heteropolymeric type O-antigen gene clusters are located between the *hemH* (ferrochelatase) and *gsk* (inosine-guanosine kinase) genes in the chromosome (Fig. 40 and Table 32). This location is different from that in *E. coli* and *S. enterica* where the O-antigen region is closely linked to the *gnd* (6-phosphogluconate dehydrogenase) locus, which is upstream of the *his* (histidine biosynthesis) operon at 45 min on the chromosomal map of *E. coli* K-12 and at centisome 45 on the *S. enterica* LT2 map (Berlyn et al., 1996; Sanderson et al., 1996).

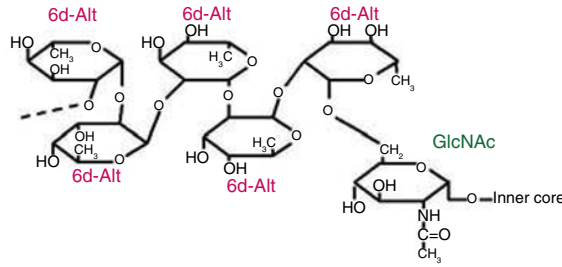


Fig. 37. O-antigen. Chemical structure of *Y. enterocolitica* O:3 homopolymeric O-antigen (6d-altrose) and its linkage via the *N*-acetylglucosamine residue to inner core.

Upstream of the *hemH* gene and the *Yersinia* O-antigen gene clusters is located the *adk* (adenylate kinase) gene, and downstream of the *gsk* gene, the *rosAB* and *ushA* (UDP-sugar hydrolase) genes, which are not directly involved in O-antigen biosynthesis. However, *rosAB* genes seem to be involved in regulation of O-antigen expression. For comparison and evolutionary considerations, in *E. coli* and *Salmonella*, the *adk*, *hemH*, *gsk* and *ushA* genes are located at 11

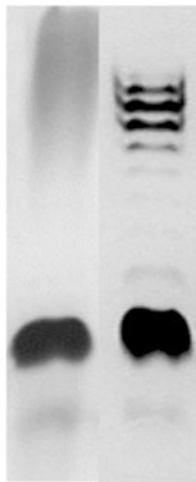


Fig. 38. Lipopolysaccharides (LPSs). Examples of LPS with homopolymeric (left) and heteropolymeric (right) O-antigens.

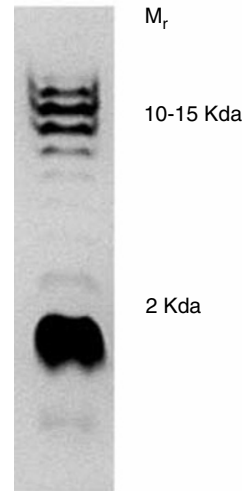


Fig. 39. Deoxycholate polyacrylamide gel electrophoretic (DOC-PAGE) analysis of lipopolysaccharide (LPS). Example of silver-stained LPS showing the heterogeneity of the LPS molecule population.



Fig. 40. The O-antigen gene cluster of *Y. pseudotuberculosis* O:1b. The gene cluster is located between the *hemH* and *gsk* genes and contains the genes for the biosynthesis of CDP-Par (*ddhDABC* and *pri*), GDP-Man (*manBC*) and GDP-L-Fuc (*gmd* and *fcl*). The *wbyIJKL* genes code for glycosyltransferases, *wzx*, *wzy* and *wzz*, for flippase, O-antigen polymerase, and O-antigen chain length determinant, respectively. Note that the cryptic O-antigen gene cluster of *Y. pestis* is almost identical to that of O:1b. From Skurnik et al. (2000).

Table 32. Sequences of LPS gene clusters of *Yersinia*.

<i>Yersinia</i> species	Cluster	Genomic location	References	GenBank acc. no.
<i>Y. enterocolitica</i> O:3	Outer core	<i>hemH</i> – <i>gsk</i>	Skurnik et al., 1995	Z47767
<i>Y. enterocolitica</i> O:3	O antigen	Unknown	Zhang et al., 1993	Z18920
<i>Y. enterocolitica</i> O:8	O antigen	<i>hemH</i> – <i>gsk</i>	Zhang et al., 1996, 1997	U46859
<i>Y. enterocolitica</i> O:8	O antigen, 3' end	<i>hemH</i> – <i>gsk</i>	Pierson and Carlson, 1996	U43708
<i>Y. pseudotuberculosis</i> O:1a	O antigen, <i>ddh</i> genes	<i>hemH</i> – <i>gsk</i>	Hobbs and Reeves, 1995	U29692
<i>Y. pseudotuberculosis</i> O:1b	O-antigen	<i>hemH</i> – <i>gsk</i>	Skurnik et al., 2000	AJ251712
<i>Y. pseudotuberculosis</i> O:2a	O antigen, <i>ddh</i> genes + 3' end	<i>hemH</i> – <i>gsk</i>	Hobbs and Reeves, 1995	L01777, U13685
<i>Y. pseudotuberculosis</i> O:4a	O antigen, <i>ddh</i> genes	<i>hemH</i> – <i>gsk</i>	Hobbs and Reeves, 1995	U29691
<i>Y. pseudotuberculosis</i> O:5a	O antigen, <i>ddh</i> genes	<i>hemH</i> – <i>gsk</i>	Hobbs and Reeves, 1995; Thorson et al., 1994	S72887
<i>Y. pestis</i> EV-76, Medievalis	O antigen, cryptic	<i>hemH</i> – <i>gsk</i>	Skurnik et al., 2000	AJ251713
<i>Y. pestis</i> CO92, Orientalis	Whole genome	<i>hemH</i> – <i>gsk</i>	<a href="http://www.sanger.ac.uk/Projects/Y_pestis/">http://www.sanger.ac.uk/Projects/Y_pestis/</a>	NC_003143

Abbreviations: LPS, lipopolysaccharide; *ddh*, dideoxyhexose gene; *hemH*, ferrochelataase gene; and *gsk*, the inosine-guanosine kinase gene.

min on the chromosome (Berlyn et al., 1996; Sanderson et al., 1996), and between *hemH* and *gsk*, these species carry only a single gene; in *E. coli*, the gene is *aes* coding for acetyl esterase (Peist et al., 1997).

*The Yersinia Outer-core Gene Clusters are also Between hemH and gsk* *Yersinia enterocolitica* serotype O:3 expresses a homopolymeric O-antigen; however, the O-antigen gene cluster is not located between the *hemH* and *gsk* genes (Zhang et al., 1993). Instead, this locus is occupied by the outer-core gene cluster (Skurnik et al., 1995). In *Y. enterocolitica* O:9 (a homopolymeric O-antigen), the outer core gene cluster is also present between the *hemH* and *gsk* genes (M. Skurnik, unpublished observation).

*The Genomic Locations of the Homopolymeric O-antigen Gene Clusters are Unknown* Thus far the only sequenced homopolymeric cluster is that of O:3 (Zhang et al., 1993) (Table 32).

For details of the LPS genes and LPS biosynthesis in specific cases, follow the links: Link 4: *Y. enterocolitica* serotype O:3 LPS, Link 5: *Y. enterocolitica* serotype O:3 outer core, Link 6: *Y. enterocolitica* serotype O:3 O-antigen, Link 7: *Y. enterocolitica* serotype O:8 LPS, Link 8: *Y. enterocolitica* serotype O:8 O-antigen, Link 9: *Y. pseudotuberculosis* LPS).

**REGULATION OF LPS BIOSYNTHESIS** *JUMPstart and ops Sequences* The gene clusters for *Y. enterocolitica* O:3 outer core and O-antigen, *Y. enterocolitica* O:8 O-antigen, and *Y. pseudotuberculosis* O-antigen, carry a characteristic sequence called “JUMPstart sequence.” The JUMPstart sequence has been identified in all surface-polysaccharide biosynthesis gene clus-

ters and also for sex-pilus assembly and hemolysin production (Hobbs and Reeves, 1994; Nieto et al., 1996). The sequence is generally located downstream of functional promoters of the gene cluster. Nieto et al. reported that a short 8-bp sequence within the JUMPstart sequence plays a role in gene regulation, and this element was named “ops” (operon polarity suppressor). The ops element was shown to function as a cis-acting element that together with RfaH (a transcription antitermination factor that stimulates expression of LPS biosynthesis genes), F-factor sex pilus genes, and hemolysin genes, allows efficient transcription of long operons, especially of the distal genes (Bailey et al., 1996). It is postulated that ops endows RfaH with specificity for the subset of operons it is regulating. The role of ops and RfaH in regulation of *Yersinia* LPS operons has not yet been studied but they are very likely to be involved.

#### *Temperature and Other Environmental Stimuli*

A general feature of *Yersinia* is temperature-dependency of the LPS biosynthesis. Most striking is the almost complete downregulation of O-antigen expression in bacteria grown at 37°C; another is the modification of the lipid A structure with respect to its substitutions such as phosphate, aminoarabinose and acyl chains (Lakshmi et al., 1989; Aussel et al., 2000). Details of the regulatory mechanisms are not known.

*Regulation of O:8 O-antigen Biosynthesis* The expression of *Y. enterocolitica* O:8 O-antigen is similarly regulated by temperature such that more O-antigen is expressed on the cell surface at 25 than at 37°C. The regulation mechanism involves repression of the O-antigen cluster transcription at 37°C (Zhang et al., 1995). The *rosAB*



locus downstream of the O-antigen cluster, between the *gsk* and *ushA* genes is involved in the temperature regulated expression of O:8 O-antigen by an unknown mechanism (Zhang et al., 1995; Bengoechea and Skurnik, 2000). RosA is an efflux pump and RosB a K<sup>+</sup>/H<sup>+</sup> antiporter (Bengoechea and Skurnik, 2000).

#### YERSINIA LPS AND VIRULENCE

**LPS mutants** No experiments on the role of LPS in the virulence of *Y. pseudotuberculosis* have been reported. On the contrary, a number of LPS mutants have been constructed in *Y. enterocolitica* strains and tested for virulence in animal experiments (Table 33). These include both O-antigen negative (rough) and outer core negative mutants. The results indicate that LPS mutant strains, either missing O-antigen or outer core, are attenuated. In signature-tagged-mutagenesis screens, mutations in the O-antigen gene cluster of *Y. enterocolitica* serotype O:8 were frequent, indicating also the critical role O-antigen plays during infection (Young and Miller, 1997; Darwin and Miller, 1999; Gort and Miller, 2000).

The LD<sub>50</sub> values for the rough *Y. enterocolitica* mutants were determined using intragastrically infected DBA/2 mice. All the mutants were less virulent than the wildtype strain; the LD<sub>50</sub> values for the rough mutants are about 50–100 times higher (Al-Hendy et al., 1992; Zhang et al., 1997); the outer core mutants are almost avirulent (Skurnik et al., 1999b). The outer core endowed the bacteria with resistance to cationic bactericidal peptides, and in coinfection experiments with the wildtype bacteria, the outer core mutant bacteria did not survive in deeper tissues as well as the wildtype bacteria, but they could colonize the Peyer's patches for 2–5 days. Similar coinfection experiments with rough mutants indicated that O-antigen is required for the initial colonization of the Peyer's patches (Skurnik, 1999a).

**Biological Activities** The endotoxic activity of *Yersinia* LPS has been addressed in some reports (Brubaker, 1972; Hartiala et al., 1989), but the role of *Yersinia* LPS in bacterial pathogenesis has received very little attention, and thus details of its biological role are far from being known. LPS isolated from different *Yersinia* species show differences in acyl-chain fluidity that correlate with OM permeability to hydrophobic agents (Bengoechea et al., 1998). Complete LPS also plays an important role in resistance against cationic antimicrobial peptides (Skurnik et al., 1999b). There are indications that O-antigen may play a role in resistance to complement-mediated killing (Wachter and Brade, 1989); however, LPS may also play some roles in adhesion to and invasion

of the host tissues and in the pathogenesis of reactive arthritis (Granfors et al., 1989; Granfors et al., 1998). LPS isolated from *Y. pseudotuberculosis* grown at 37°C, when integrated into artificial membranes, protects membrane vesicles from the complement activity (Porat et al., 1995). Passive immunization has been used to show that antibodies against the O-antigen, but not those against the core, protect mice against intravenous challenge of virulent bacteria (Skurnik et al., 1996).

#### Superantigens of *Y. pseudotuberculosis*

Superantigens are proteins that exhibit a highly potent polyclonal lymphocyte-proliferating activity for CD<sub>4</sub><sup>+</sup>, CD<sub>8</sub><sup>+</sup> and sometimes  $\gamma\delta$ <sup>+</sup> T lymphocytes. Unlike conventional antigens, superantigens bind as unprocessed proteins to the major histocompatibility complex (MHC) class II molecules present on the surface of antigen-presenting cells and to the variable region of the  $\beta$  chain (V $\beta$ ) of T-cell receptor (TcR; Marrack and Kappler, 1990). As a consequence, superantigens stimulate up to 30% of T cells and trigger the release of large amounts of pro-inflammatory and other cytokines, which cause deleterious effects in the host (Immunology). As they stimulate increased numbers of lymphocytes, superantigens may stimulate autoreactive T and B cells and thus initiate or worsen autoimmune diseases (Ulrich et al., 1995; Michie and Cohen, 1998).

Until the beginning of the 1990s, bacterial superantigens had only been identified in *Mycoplasma arthritidis* and the Gram-positive species *Staphylococcus aureus* and *Streptococcus pyogenes*. In 1993, it was discovered that a superantigen was released into the culture supernatant of *Y. pseudotuberculosis* strains (Abe et al., 1993; Miyoshi-Akiyama et al., 1993; Uchiyama et al., 1993). Interestingly, one of the producers was isolated from a patient with the clinical manifestations of Kawasaki syndrome, a systemic vasculitis of unknown cause and a pathophysiology that might involve a superantigen (Leung et al., 1995; Clinical manifestations of *Y. pseudotuberculosis* infections). The substance, purified from *Y. pseudotuberculosis* culture supernatants and inducing proliferation of human peripheral blood mononuclear cells at a concentration as low as 1 pg ml<sup>-1</sup>, was designated "YPM" (for *Y. pseudotuberculosis*-derived mitogen). Cloning and sequencing of the YPM gene revealed a 456-base pair ORF that encodes a 151 amino acid precursor protein. After proteolytic cleavage of the 20 amino acid hydrophobic signal sequence, this precursor is processed into a mature form containing 131 residues (Yoshino et al., 1994a; Ito et al., 1995; Miyoshi-Akiyama et al., 1995; Abe and Takeda,

Table 33. Role of *Yersinia* LPS in virulence. Results of animal experiments.

Species	Strain	Mutation	Phenotype	Animal host	Experiment	Results	References
<i>Y. enterocolitica</i> O:3	YeO3-R2	O-antigen cluster	Rough	Mouse	i.g. LD <sub>50</sub>	50-fold attenuation	Al-Hendy et al., 1992
	YeO3-R2	O-antigen cluster	Rough	Mouse	Coinfection	Attenuated, does not colonize or is eliminated from Peyer's patches within 2d, not found in deeper tissues	Skurnik et al., 1999
	YeO3-trs11	$\Delta wzx wbcK wbcL$	Outer core	Mouse	i.g. LD <sub>50</sub>	Avirulent	Skurnik et al., 1999
	YeO3-trs11	$\Delta wzx wbcK wbcL$	Outer core	Mouse	i.v. LD <sub>50</sub>	10 <sup>4</sup> -fold attenuation	Skurnik et al., 1999
<i>Y. enterocolitica</i> O:8	YeO3-trs22	$\Delta wbcKL$	Outer core	Mouse	i.g. LD <sub>50</sub>	Avirulent	Skurnik et al., 1999
	YeO3-trs24	$\Delta wbcP$	Outer core	Mouse	i.g. LD <sub>50</sub>	Avirulent	Skurnik et al., 1999
	YeO3-trs24	$\Delta wbcP$	Outer core	Mouse	Co-infection	Attenuated, persists in Peyer's patches for 2–5d, not found in deeper tissues	Skurnik et al., 1999
	6471/76	Wild-type	Wild-type	Mouse	Passive immunization with anti-O-Ag Mab. i.v. challenge	Good protection	Skurnik et al., 1999
<i>Y. enterocolitica</i> O:8	6471/76	Wild-type	Wild-type	Mouse	Passive immunization with anti-core Mab. i.v. challenge	No protection	Skurnik et al., 1996
	8081-R2	O-antigen cluster	Rough	Mouse	i.g. LD <sub>50</sub>	100-fold attenuation	Zhang et al., 1997
	8081 (signature tagged mutagenesis library)	Insertions in the O-antigen gene cluster	Rough	Mouse	STM screen, coinfection	Attenuation	Darwin and Miller, 1999; Gort and Miller, 2000; Young and Miller, 1997

Abbreviations: 50% lethal dose; i.v., intravenously; i.g., intragastrically; Mab, monoclonal antibody; and STM, signature tagged mutagenesis.

1997a). YPM does not share any homology with staphylococcal, streptococcal or mycoplasmal superantigens, and phylogenetic analysis clearly demonstrated that the *Y. pseudotuberculosis* superantigen is novel (Müller-Alouf et al., 2001). After the initial discovery of YPM, two variants were described: one (one amino acid shorter) is 83% identical to the original molecule, the most heterogeneous region lying between amino acid residues 65 and 86 (Ramamurthy et al., 1997a), whereas the other one differs only by a single amino acid substitution (His51 → Tyr; Carnoy and Simonet, 1999). These variants are now known as “YPMb” and “YPMc,” respectively, and the original superantigen is called “YPMa” (GenBank accession numbers: {D38523} and {D38638} for *ypmA*; {D88144} for *ypmB*). In 1992, the other enteropathogenic *Yersinia* species, *Y. enterocolitica*, was shown to produce superantigen (Stuart and Woodward, 1992). However, the T-cell specificity of the mitogen differs from that of YPM (Stuart et al., 1995) and genomic bacterial DNA could not hybridize to a *ypm* probe (Yoshino et al., 1995a; Carnoy and Simonet, 1999) indicating that the mitogen activity of *Y. enterocolitica* could not be attributed to *ypm*. The mitogenic substance of *Y. enterocolitica* has not yet been characterized. Surprisingly, *ypm* was not detected in *Y. pestis* (Miyoshi-Akiyama et al., 1995), although this species is genetically closely related to *Y. pseudotuberculosis*.

In vitro, the three YPM variants all selectively stimulate human T lymphocytes bearing  $V_{\beta 3}$ ,  $V_{\beta 9}$ ,  $V_{\beta 13.1}$  and  $V_{\beta 13.2}$  TcR variable regions, in an MHC class II-dependent manner (Abe et al., 1993; Uchiyama et al., 1993; Ramamurthy et al., 1997a). YPM also stimulates murine T-cells bearing  $V_{\beta 7}$  and  $V_{\beta 8}$  (Miyoshi-Akiyama et al., 1997). Both  $CD_4^+$  and  $CD_8^+$  cells are expanded, but  $CD_8^+$  cells proliferate predominantly (Ito et al., 1995). YPM is a small superantigen (14.5 kDa) compared to the known bacterial superantigens, the size of which ranges from 20–30 kDa. The molecular mechanism by which YPM interacts with its two ligands, TcR and MHC class II molecules, is poorly known. By testing the blocking effect of synthetic peptides on YPM-triggered lymphocyte proliferation, it was found that a peptide corresponding to the first 23 amino acids of the *Y. pseudotuberculosis* superantigen was the best inhibitor; however, this peptide corresponding to the N-terminus region of the molecule only inhibited cell proliferation by 50% (Yoshino et al., 1996). Subsequently, mutational analysis of YPM revealed that the amino acids involved in ligand interactions are in fact scattered along the polypeptide chain rather than clustered into domains, and that two cysteine residues, located at positions 32 and 129, form a

disulfide bridge that is critical for the biological activity of YPM (Ito et al., 1999).

The superantigen-encoding gene is not present in all *Y. pseudotuberculosis* strains, and its distribution is independent of the O-serotype (Carnoy and Simonet, 1999). Interestingly, almost all isolates from the Far East and only one-fifth of European isolates were positive for *ypm* (Yoshino et al., 1995a; Carnoy and Simonet, 1999). In the Far East, human *Y. pseudotuberculosis* infection is frequently associated with a scarlet fever-like disease, Izumi fever, or Kawasaki-like syndrome (Somov and Martinevsky, 1973; Sato et al., 1983; Baba et al., 1991), which are seldom described in Europe and North America (Epidemiology of *Y. pseudotuberculosis*). However, there is currently no evidence of a correlation between these particular clinical manifestations and the presence of *ypm* in infecting strains.

YPM is produced by bacteria during the infectious process in the host: indeed, 1) anti-YPM antibodies are present in the blood of most humans infected with *Y. pseudotuberculosis*, with a higher titer in patients with systemic complications, and 2) the number of T cells bearing  $V_{\beta 3}$  (but not  $V_{\beta 9}$ ,  $V_{\beta 13.1}$  and  $V_{\beta 13.2}$ ) increases during the acute phase of the yersiniosis (Abe et al., 1997b). Furthermore, it was found that purified YPM (50  $\mu$ g) can induce lethal shock in a mouse experimental model by producing tumor necrosis factor (TNF- $\alpha$ ) and interferon  $\gamma$  (Miyoshi-Akiyama et al., 1997). The toxin also alters epithelial function in vitro by reducing active ion transport and increasing cellular permeability (Donnelly et al., 1999). Taken together, these results suggested a role for YPM in *Y. pseudotuberculosis* pathogenesis. In vivo comparison of the virulence of a wildtype strain and an isogenic mutant provided more relevant information in this field because this experimental approach evaluated the effect of the superantigen in the host in the context of the whole microorganism. Furthermore, the use of living *Yersinia* ensured the release of a physiological quantity of superantigenic toxin over a long period. In the murine model of systemic yersiniosis, the survival rate was higher for the animals infected with the superantigen-deficient strain than in animals infected with wild-type strain and lower virulence of the mutant could not be accounted for by a reduced bacterial growth in mouse tissues (Carnoy et al., 2000). The mechanisms by which YPM increases *Yersinia* virulence are not understood yet. A synergistic action of endotoxin with staphylococcal superantigen has been reported (Blank et al., 1997); therefore, one plausible explanation is that LPS effects are exacerbated by YPM.

Nucleotide sequence analysis revealed that the guanine and cytosine (G+C) content of *ypm*

was 35 mol%, whereas the G+C content of the *Y. pseudotuberculosis* genome is higher (47 mol%). This suggests that during evolution, *Y. pseudotuberculosis* obtained *ypm* from microorganisms with low G+C contents. The superantigen-producing species, *S. aureus*, *S. pyogenes* and *M. arthritis*, are good candidates. However, the codon usage of *ypmB*, which is thought to be the ancestral gene on the basis of molecular (IS1541) typing of thirty nonepidemiologically related *ypm*-positive strains of *Y. pseudotuberculosis* (Carnoy and Simonet, 1999), is different from that of *S. aureus*, *S. pyogenes* and *M. arthritis*. This indicates that the *ypm* genes came from another microbial source (Carnoy et al., 2002). The sequence of the genetic environment of the superantigen-encoding gene showed that *ypm* is not associated with other virulence genes, and that unlike many staphylococcal and streptococcal superantigen genes, it is not located on a mobile genetic element, such as a phage, a transposon or a plasmid. It is now known that the chromosomal region containing *ypm* is the site at which deletion/insertion genetic events occur. The presence of a recombination/phage integration site upstream of the superantigen gene probably contributes to the occurrence of these events (Carnoy et al., 2002). However, it is still not known how *Y. pseudotuberculosis* acquired *ypm*.

### Other Putative Virulence Factors (Elisabeth Carniel)

In addition to the well-characterized factors described above, other factors have been implicated to different extents in *Yersinia* pathogenesis, but their role often remains obscure or controversial. A few of them are presented below.

**HEAT-STABLE ENTEROTOXINS OF *Y. ENTEROCOLITICA*** Three types of heat-stable enterotoxins, designated "YSTa," "YSTb," and "YSTc," have been identified in *Y. enterocolitica* until now.

**YSTa Mode of Action** The presence of a heat-stable methanol-soluble enterotoxin YST (now YSTa) produced by *Y. enterocolitica* was initially detected in the infant-mouse test (Pai and Mors, 1978; Boyce et al., 1979; Feeley et al., 1979; Okamoto et al., 1980). Its structure and mode of action are similar to those of the heat-stable methanol-soluble toxin STI (for stable toxin I) of enterotoxigenic *E. coli* (Pai and Mors, 1978; Boyce et al., 1979; Feeley et al., 1979; Robins-Browne et al., 1979b). This enterotoxin activates the particulate form of intestinal guanylate

cyclase and induces fluid accumulation by increasing the concentration of cyclic GMP levels within intestinal epithelial cells (Rao et al., 1979; Robins-Browne et al., 1979b; Inoue et al., 1983; Inoue et al., 1984b; Inoue et al., 1986).

**Structure** The chromosomal *ystA* gene encodes a 71-amino-acid (AA) product (Delor et al., 1990). The mature 30-AA enterotoxin found in supernatants is a processed form obtained after removal of the N-terminal 19-amino acid (AA) signal sequence (pre-domain) and cleavage of the central 22-AA pro-domain during or after secretion (Takao et al., 1984; Takao et al., 1985; Delor et al., 1990). This organization in pre-, pro- and mature YSTa is reminiscent of that of the *E. coli* STI. The conservation of the pre- and pro-domains between *Y. enterocolitica* and *E. coli* is moderate and the size of the mature toxin is much larger in the former (30 AA) than in the latter (18 AA) species (Delor et al., 1990). Heat-stable enterotoxins (STs) produced by various enteric bacteria share a highly conserved sequence formed of 13 AA with 6 Cys residues linked by 3 disulfide bonds. This sequence, which constitutes the toxic domain of the peptide and is highly conserved among heat-stable toxins, is also present in YSTa (Takao et al., 1985; Delor et al., 1990).

**Regulation** In vitro, maximal synthesis of YSTa occurs at 26°C, during the late phase of growth (Boyce et al., 1979; Amirmozafari and Robertson, 1993) and is due to a transcriptional regulation of *ystA* (Mikulskis et al., 1994; Nakao et al., 1994; Nakao et al., 1995). However, transcription of *ystA* could still be induced at 37°C by increasing osmolarity and pH to the values normally present in the ileum lumen (Mikulskis et al., 1994), suggesting that the enterotoxin may be produced under physiological conditions. Two global regulators, the histone-like negative regulator YmoA (Mikulskis et al., 1994) and the *yrp* (*Yersinia* regulator for pleiotropic phenotype) locus homologous to the *hfq* gene encoding a host factor protein of *E. coli* (Nakao et al., 1994; Nakao et al., 1995), participate in this temperature and growth phase regulation.

Although fresh clinical isolates of *Y. enterocolitica* generally produce YSTa, silencing of *ystA* is frequently observed upon storage in vitro. The two regulators YmoA and Yrp also participate in the switching on and off of *ystA*. YmoA is involved in *ystA* silencing, and a mutation in *ymoA* reactivates the production of YSTa (Cornelis et al., 1991; Mikulskis et al., 1994). By contrast, *yrp* is necessary for *ystA* expression. Various mutations in *yrp* were found in *Y. enterocolitica* strains that had a silent *ystA* gene, and complementation of these strains with an active

*yrp* locus restored YST production (Nakao et al., 1994; Nakao et al., 1995).

**Role in Pathogenicity** The role of YSTa in the pathogenesis of *Y. enterocolitica* diarrhea in humans remains controversial. A *Y. enterocolitica* O:3 strain that did not produce YSTa was still capable of producing diarrhea in orally infected mice (Schiemann, 1981a). In a gnotobiotic newborn piglet model, a *Y. enterocolitica* O:3 *yst* mutant remained virulent (Robins-Browne et al., 1985b). Furthermore, no enterotoxin production was detected in feces or intestines of mice, rabbits, or piglets with *Y. enterocolitica*-induced diarrhea (Pai et al., 1980; Schiemann, 1981a; Robins-Browne et al., 1985b). In contrast, in a model of orally infected young rabbits, a *ystA* mutant of *Y. enterocolitica* O:9 lost the ability to cause diarrhea, weight loss, and death of the animals. The mutant was also eliminated more rapidly from the feces than the YSTa-producing parental strain (Delor and Cornelis, 1992).

The demonstration that 100% of pathogenic versus 0% of nonpathogenic *Y. enterocolitica* harbored the *ystA* locus (Delor et al., 1990; Robins-Browne et al., 1993) was also an argument for a role of YSTa in pathogenesis. However, a few strains belonging to the nonpathogenic species *Y. kristensenii* and *Y. intermedia* were also found to possess the *ystA* locus (Delor et al., 1990; Ramamurthy et al., 1997b).

**YSTb** Investigation of a large number of *Y. enterocolitica* isolates for the presence of the *ystA* locus and the production of an enterotoxigenic activity revealed that some strains did produce an enterotoxin despite the absence of an *ystA* gene (Robins-Browne et al., 1993; Yoshino et al., 1994b). A novel enterotoxin, designated "YSTb," was purified from the supernatant of a *Y. enterocolitica* O:5 strain and characterized. This new toxin was found to be 20-fold more potent in the suckling mouse assay than YSTa (Yoshino et al., 1994b). This higher toxicity was attributed to the substitution of Trp<sup>17</sup> for Asp<sup>17</sup>, and of Glu<sup>20</sup> for Asp<sup>20</sup> (Yoshino et al., 1994b) in YSTb. The amino-acid sequence of the 30-AA mature form of YSTb differed from that of YSTa in the N-terminal part of the peptide (AA 1–17) but was similar in its C-terminal portion (AA 18–30; Yoshino et al., 1994b).

The *ystB* gene was subsequently cloned and found to be 73.5% identical to *ystA*. It had the potential to encode a 71-AA long peptide (Ramamurthy et al., 1997b). Screening of a large number of *Y. enterocolitica* isolates for the presence of *ystB* indicated that this gene is present in 11.8% of isolates, which all belonged to the nonpathogenic group of biotype 1A (Ramamurthy et al., 1997b).

**YSTc** Another novel heat-stable enterotoxin, designated "YSTc," was identified from an enterotoxigenic *Y. enterocolitica* O:3 strain, which failed to hybridize with *ystA* (Yoshino et al., 1995b). In contrast to YSTa and YSTb, the mature form of YSTc was very long (53 AA), representing the largest molecular size toxin in the family of currently known STs. This secreted peptide was composed of the pro- and mature domain of the polypeptide (Yoshino et al., 1995b). The presence of the pro-domain in the secreted mature toxin suggests that in *Y. enterocolitica*, the proteolytic processing of the pro-domain is not essential for the secretion of YSTc to the extracellular compartment through the outer membrane. The toxic domain in the C-terminal portion of YSTc was well conserved (Yoshino et al., 1995b).

In contrast to YSTa, YSTb is produced in cells grown at 37°C (Yoshino et al., 1995b). YSTc was found to have an enterotoxigenic activity four times superior to those of YSTb and STb of *E. coli*, which had been known as the most potent STs. Therefore, YSTc may be considered as the most potent enterotoxin in the ST family (Yoshino et al., 1995b).

The *ystC* gene is 73.1% and 73.5% identical to *ystA* and *ystB*, respectively (Huang et al., 1997; Ramamurthy et al., 1997b) and encodes a 72-AA product (Huang et al., 1997). Screening of a large number of *Y. enterocolitica* isolates for the presence of *ystC* indicated that this gene is present in a very low number of isolates (1%) belonging either to the pathogenic or the nonpathogenic group (Ramamurthy et al., 1997b).

**Conclusion** To date, three different heat-stable enterotoxins have been identified in *Y. enterocolitica*. The high degree of identity between these three enterotoxins indicates that they should not be considered as different STs but as different subtypes of YST (Yoshino et al., 1994b). The fact that none of the *Y. enterocolitica* strains tested hybridized with more than one probe (Ramamurthy et al., 1997b) may indicate a mutual exclusion of these three gene subtypes in *Y. enterocolitica*. Finally, the existence of pathogenic (and therefore diarrheagenic) strains that do not harbor any of the three known enterotoxigenic genes (Ramamurthy et al., 1997b) suggests that additional, yet unidentified, enterotoxins may be produced by *Y. enterocolitica*.

**FIMBRIAE** Pathogenic *Yersinia* synthesize a fibrillar structure at their surface, designated "Myf" (mucoid *Yersinia* factor) in *Y. enterocolitica* and pH6 antigen (or Psa) in *Y. pseudotuberculosis* and *Y. pestis*. Although these two fimbriae may not necessarily play the same role and seem to have diverged more than other virulence factors

during evolution, they share some common epitopes. They are produced in the same environmental conditions, and their chromosomal loci display a similar genetic organization and some degree of nucleotide identity (Iriarte et al., 1993; Lindler and Tall, 1993; Iriarte and Cornelis, 1995; Leiva et al., 1995).

*Properties of Myf in Y. enterocolitica* The Myf antigen was originally described as a 24-kDa protein loosely associated with the bacterial membrane (Diaz et al., 1985). Synthesis of this antigen was obtained only when the bacteria were grown on solid media at 37°C, in the presence of a metabolizable sugar. Immunogold labeling subsequently revealed that Myf forms a fibrillar structure extending 2 µm from the cell surface (Iriarte et al., 1993).

Although never demonstrated, the role of Myf in *Y. enterocolitica* pathogenesis was suggested by the observations that only pathogenic serotypes of *Y. enterocolitica* produced these fimbriae (Diaz et al., 1985; Toyos et al., 1986) and harbored the pilin subunit-encoding gene *myfA*. However, the presence of the *myfA* locus was recently detected in some *Y. enterocolitica* strains of biotype 1A (Grant et al., 1998), which are conventionally considered as nonpathogenic.

*Properties of pH6 Antigen in Y. pseudotuberculosis and Y. pestis* The pH6 antigen (Ag) received its name following the observation that a specific antigenic surface component was produced by various strains of *Y. pestis* after incubation at 37°C and at pH6 (Ben-Efraim et al., 1961). This antigen was subsequently identified in *Y. pseudotuberculosis* (Ben-Efraim and Bichowsky-Slomnichi, 1964; Lindler and Tall, 1993) and found to be highly conserved, both genetically and immunologically, between the two species (Lindler and Tall, 1993). However, while all strains of *Y. pestis* produced pH6 Ag, only some isolates of *Y. pseudotuberculosis* appeared to do so (Ben-Efraim and Bichowsky-Slomnichi, 1964; Lindler and Tall, 1993).

Electron microscopy and immunogold labeling demonstrated that pH6 Ag forms fibrillar organelles that protrude from the cell surface as subtle, fine, singular strands (ca. 4 nm in diameter), as multistranded bundles, or as large aggregates in *Y. pestis*, *Y. pseudotuberculosis* and *E. coli* clones carrying the *psa* locus (Lindler and Tall, 1993).

Crude extracts of *Y. pestis* pH6 Ag were initially found to be cytotoxic for monocytes, to agglutinate erythrocytes, and to induce inflammatory reactions of the skin (Bichowsky-Slomnicky and Ben-Efraim, 1963). The hemagglutination property of the *Y. pseudotuberculosis* pH6 Ag was also demonstrated (Yang

et al., 1996; Yang and Isberg, 1997). The protein appears to possess several other properties. It binds to the Fc portion of human immunoglobulin (Ig)G (but not that of rabbit, mouse, and sheep IgG) and can thus be considered as a bacterial Fc receptor (Zav'yalov et al., 1996). It mediates thermoinducible adhesion of *Y. pseudotuberculosis* to cultured epithelial cells (Yang et al., 1996). It binds the β1-linked galactosyl residues in glycosphingolipids (Payne et al., 1998b).

Several pieces of evidence indicate a role for pH6 Ag in *Y. pestis* pathogenesis: 1) antibodies against pH6 Ag were found in the sera of rabbits infected with various *Y. pestis* isolates (Ben-Efraim et al., 1961), 2) *Y. pestis* cells recovered from the spleen or liver of infected mice expressed the antigen at their surface (Ben-Efraim et al., 1961), 3) the rate of mouse mortality was higher during the first 4 days postinfection when the animals were infected with pH6 Ag-expressing cells (grown at pH6) than with pH6 Ag-nonexpressing cells (grown at pH7; Ben-Efraim et al., 1961), 4) *Y. pestis* associated with a murine macrophage-like cell line expressed pH6 Ag in an intracellular acidification-dependent manner (Lindler and Tall, 1993), and 5) mutation in the *psa* locus (*psaA* or *psaE*) resulted in a ≥100-fold increase in the LD<sub>50</sub> for mice infected retro-orbitally (Lindler et al., 1990).

*Genetic Organization of the myf and psa Loci* The genetic organizations of the Myf and pH6 Ag systems are very similar. The different components of these two systems and their putative locations and functions are summarized in [Table 34](#).

*Structural Components* PsaA/MyfA encodes the major 21-kDa subunit of the fimbriae, which are assembled at the bacterial surface by the transport and assembly machinery composed of PsaB/MyfB and PsaC/MyfC (Iriarte et al., 1993; Yang and Isberg, 1997). The Myf and pH6 Ag display homology with the corresponding components of the Pap system of *E. coli* and partly with the pFra-encoded Caf system of *Y. pestis* (Iriarte et al., 1993; Lindler and Tall, 1993).

*Regulation* The *myf* (*psa*) locus is composed of two operons: *myfEF* (*psaEF*) and *myfABC* (*psaABC*). Expression of *myfABC* (*psaABC*) is regulated at the transcriptional level by temperature and pH, and requires the upstream activators *myfE/psaE* and *myfF/psaF* (Iriarte et al., 1993; Price et al., 1995; Yang and Isberg, 1997). However, *psaEF*, itself, is not regulated by pH or temperature (Lindler et al., 1990) but is constitutively expressed (Yang et al., 1996). Although

Table 34. Correspondence between the different components of the Myf and pH6 Ag systems.

<i>Y. enterocolitica</i>	<i>Y. pestis</i> <i>Y. pseudotuberculosis</i>	Putative location	Putative function
MyfF	PsaF	Inner membrane and periplasm	Activator
MyfE	PsaE	Cytoplasm, inner membrane, and periplasm	Activator
MyfA	PsaA	Surface	Subunits of fimbriae
MyfB	PsaB	Periplasm	Chaperone
MyfC	PsaC	Outer membrane	Transport and assembly

a temperature of 37°C is required for full expression of *psaABC*, pH is the initial environmental stimulus essential for expression of *psaA* (Price et al., 1995). The *myfABC* operon can be transcribed from either a classical  $\sigma 70$  or a  $\sigma 28$  promoter (Iriarte and Cornelis, 1995).

PsaE has an N-terminal cytoplasmic domain and a periplasmic C-terminal domain, and shares similarities with transcriptional regulators found in two-component systems (Yang and Isberg, 1997). PsaF/MyfF is presumably involved in transduction of the signal triggering transcription of *myfABC*. The predominant localization of PsaF/MyfF in the periplasm strikingly resembles that of ToxS, a protein involved in regulation of Tcp pilus production in *Vibrio cholerae* (Iriarte and Cornelis, 1995) and suggests that MyfF may be part of a cascade of regulation. It is thus possible that the two regulators MyfE/PsaE and MyfF/PsaF interact with each other in the periplasm and function together to sense environmental cues such as pH and temperature (Iriarte and Cornelis, 1995; Yang and Isberg, 1997).

**IRON UPTAKE SYSTEMS** The ability to capture iron in vivo through the HPI-borne yersiniabactin system is a key determinant that differentiates high- and low-pathogenicity *Yersinia* strains. In addition to this potent iron-acquisition system, *Yersinia* spp. have developed other strategies to acquire this important metal. The iron acquisition systems other than yersiniabactin are presented below.

**Role of Iron in the Pathogenesis of Enteropathogenic *Yersinia*** Iron serves as a cofactor in a number of enzymatic and metabolic pathways and represents an essential growth factor for almost all bacteria except certain lactobacilli and *Borrelia burgdorferi* (Posey and Gherardini, 2000). In mammals, this metal is bound to eukaryotic proteins (hemoglobin, ferritin, transferrin and lactoferrin), which maintain a level of free iron exceedingly too low ( $10^{-18}$  M) to sustain bacterial growth. Among bacterial pathogens, the successful establishment of disease depends

on the ability of the invading organism to acquire iron. Microbial pathogens must therefore adapt to the iron-restricted milieu found in vivo and develop high-affinity iron transport systems called “siderophores.”

Low-pathogenicity strains of *Y. enterocolitica* (biotypes 2 and 4) and *Y. pseudotuberculosis* serotypes II, IV and V do not produce their own siderophore and are usually restricted to the intestinal tract. However, these bacteria can cause systemic infections if a source of iron becomes available in vivo. Clinical case descriptions of disseminated infections due to low-pathogenicity *Yersinia* in iron-overloaded patients are numerous in the literature. This iron-overload status responsible for disseminating *Yersinia* infections had different origins: oral overdose of iron (Robins-Browne et al., 1979a; Soriano et al., 1981; Melby et al., 1982; Mofenson et al., 1987); iron therapy (Leighton and MacSween, 1987; Fakir et al., 1995); hemodialysis (Boelaert et al., 1987) and long-term transfusion therapy (Schuchmann et al., 1997); and underlying diseases that increase directly or indirectly (through blood transfusions) the iron burden of the patients:

- 1) hypersideremia without any known etiology (Sibilia et al., 1991),
- 2) primary hemochromatosis (Jacquenod et al., 1984; M'Rad et al., 1988; Shibuya et al., 1988; Merrien et al., 1991; Vadillo et al., 1994; Collazo et al., 1995; Piroth et al., 1997; Höpfner et al., 2001),
- 3) thalassemia (Blum et al., 1970; Hewstone and Davidson, 1972; Seigneurin et al., 1972; Rabson et al., 1975; Bouza et al., 1980; Hambourg et al., 1980; Soriano et al., 1981; Adamkiewicz et al., 1998),
- 4) autoimmune hemolytic anemia (Guez et al., 1987), and
- 5) liver diseases (Marlon et al., 1971; Seigneurin et al., 1972; Rabson et al., 1975; Imhoof and Auckenthaler, 1980; Soriano et al., 1981; Jacquenod et al., 1984; Merrien et al., 1991).

The role of iron in *Yersinia* pathogenicity was also demonstrated in the mouse experimental

model of infection. Low-pathogenicity *Y. enterocolitica* strains, which are naturally nonlethal for mice, gained this lethal power when exogenous iron was administered to the animals (Robins-Browne and Prpic, 1985a; Smith et al., 1981). The suppressive effect of this metal on the host immune response may partly account for the enhanced bacterial pathogenicity. Nonetheless, iron appears to play a crucial role in *Yersinia* pathogenesis.

**Utilization of Exogenous Siderophores** Several siderophores that are not synthesized by *Y. enterocolitica* and *Y. pseudotuberculosis* can still be used as a source of iron when they are supplied in the growth medium (Perry and Brubaker, 1979; Brock and Ng, 1983; Brock et al., 1988; Rabsch and Winkelmann, 1991; Bäumler et al., 1993; Table 35).

One of the clinically most important exogenous siderophores is deferoxamine B, a licensed therapeutic substance (Desferal®) commonly used to treat patients with iron-overload (thalassemia, hemodialysis, etc.). This hydroxamate-type siderophore, naturally produced by *Streptomyces pilosus* and by various enterobacteriaceae, can be used by low-pathogenicity yersiniae for capturing iron, thus enhancing their ability to disseminate in a mouse host (Robins-Browne and Prpic, 1985a). Clinical reports of deferoxamine-treated patients that developed fulminant *Y. enterocolitica*-disseminated infections are numerous (Kiffer et al., 1975; Butzler et al., 1978; Di Palma et al., 1982; Roche et al., 1982; Robins-Browne and Prpic, 1983; Aristegui et al., 1985; Boyce et al., 1985; Waterlot and Vanherweghem, 1985; Gallant et al., 1986; Trallero et al., 1986; Francois et al., 1987; Warren, 1987; Hoen et al., 1988; Chirio et al., 1990; Cianciulli et al., 1990; Abcarian and Demas, 1991; Mazzoleni et al., 1991; Pacifico et al., 1991; Hoe et al., 1994; Cherchi et al., 1995; Paitel et al., 1995; Adamkiewicz et al., 1998). This virulence enhancing effect of deferoxamine has at least two causes. One is the synthesis by *Y. enterocolitica*

of the outer-membrane protein FoxA that acts as a receptor for the exogenous siderophore (Bäumler and Hantke, 1992). The other is the modulation/abolition of the action of specific and nonspecific immune cells and the inhibition of cytokine production by macrophages, leading to partial immunosuppression of the host (Autenrieth et al., 1991; Autenrieth et al., 1994b; Autenrieth et al., 1995). Thus, low-pathogenicity *Y. enterocolitica* strains usually restricted to the digestive tract can use deferoxamine to obtain the limiting iron molecules and benefit from the induced-immune deficiency to disseminate in their host and cause systemic infections.

To utilize exogenous siderophores, pathogenic *Yersinia* need to express the specific receptors on their surface and/or the appropriate transporters across their two membranes. Several of these receptors have been identified in *Yersinia* (Table 35). They all possess a TonB box and their synthesis is induced under iron-stress conditions (Bäumler and Hantke, 1992; Koebnik et al., 1993a; Koebnik et al., 1993b).

Analysis of the membrane topology of the deferoxamine receptor FoxA indicates that this protein forms a barrel consisting of 32 trans-membrane  $\beta$ -pleated sheets (Bäumler and Hantke, 1992; Bäumler et al., 1993). While FoxA displays an exclusive specificity for deferoxamine B, the ferrichrome receptor FcuA can transport almost all ferrichrome derivatives with equal efficiency (Koebnik et al., 1993b). Both FoxA and FcuA carry a Fur box, suggesting that they are both negatively regulated by this protein in the presence of iron (Bäumler and Hantke, 1992; Koebnik et al., 1993b). A mutation in *foxA* or *fcuA* does not alter the virulence of *Y. enterocolitica*.

The enterochelin receptor FepA has been recognized by monoclonal antibodies raised against *E. coli* FepA as a 90-kDa protein in *Y. enterocolitica* membrane extracts (Rutz et al., 1991). Curiously, in some *Y. enterocolitica* strains (serotype O:8 and O:20), the *fepA* gene contains a frameshift mutation that leads to a truncated

Table 35. Exogenous siderophores used by enteropathogenic *Yersinia*.

Siderophore	<i>Y. enterocolitica</i>	<i>Y. pseudotuberculosis</i>	Receptor	Molecular mass (kDa)	References
Ferrichrome	+	UN	FcuA	78	Koebnik et al., 1993
Aerobactin	+/-	+	UN	UN	NA
Schizokinen	+	+	UN	UN	NA
Ferrioxamine	+/-	+/-	FoxA	75.7	Bäumler and Hantke, 1992
Enterochelin	+/-	+/-	FepA <sup>a</sup>	83	Rutz et al., 1991; Schubert et al., 1999
Catecholate	+	UN	CccA	65	Bäumler et al., 1993

Symbols and abbreviations: +, present in all strains; -, absent in all strains; +/-, present in most strains; UN, unknown, and NA, none available.

<sup>a</sup>*fepA* gene mutated in some strains.



peptide, while in other strains (serotype O:13 and O:5), a 31-bp insertion leads to the restoration of a single ORF (Schubert et al., 1999a). Genes involved in enterochelin transport (*fep-DGC*) and in the release of iron from enterochelin (*fes*) in the cytosol are present and functional in *Y. enterocolitica* O:8, while those involved in enterochelin biosynthesis (*ent*) are absent from the bacterial genome (Schubert et al., 1999a).

**Iron Uptake from Heme-Proteins** Another pathway used by *Yersinia* to capture iron is to utilize the iron molecules bound to heme-proteins. A locus involved in hemin uptake (*hem*) has been characterized in *Y. enterocolitica* (Stojiljkovic and Hantke, 1992; Stojiljkovic and Hantke, 1994). This locus is homologous to the hemin locus uptake (*hmu*) of *Y. pestis* (86–93% AA identity; Thompson et al., 1999) and allows the utilization of a wide range of heme-proteins as sources of iron (Bracken et al., 1999). In addition to iron, some heme-proteins may also be used as a source of porphyrin by *Yersinia* (Stojiljkovic and Hantke, 1992).

The *hem* locus is composed of at least six genes forming two operons: *hemPR* and *hemSTUV*, and is iron- and Fur-regulated (Stojiljkovic and Hantke, 1994). The location and putative functions of the products of this locus are listed in Table 36. HemR is the TonB-dependent iron-regulated surface receptor for heme. Its membrane topology probably consists in 22 trans-membrane  $\beta$ -strands and 11 surface-exposed loops (Bracken et al., 1999). Two histidine residues (His<sup>128</sup> and His<sup>461</sup>) are thought to participate in the transport of heme through the HemR pore (Bracken et al., 1999). A model has been proposed in which the HemTUV complex transports hemin into the bacterial cytoplasm where it is degraded by HemS, thereby liberating the iron molecules and preventing the toxic effect of heme (Stojiljkovic and Hantke, 1994). The HemSTUV system of heme transport across the inner membrane is similar to the siderophore-uptake systems. HemP is not essential for hemin-porphyrin or hemin-iron utilization (Stojiljkovic and Hantke, 1994).

Interestingly, the *hem* locus is located close to the *foxA* gene that codes for the ferrioxamine

receptor, forming a chromosomal “iron island.” Since a *hemR* mutant strain of *Y. enterocolitica* retains its pathogenicity upon intravenous injection, heme capture does not seem to be crucial for bacterial survival in vivo (Stojiljkovic and Hantke, 1992).

The heme-uptake system has not yet been studied in *Y. pseudotuberculosis*. However, the strong hybridization signal obtained with an *hmu* probe suggests that a similar system exists in this species (Thompson et al., 1999).

#### *Siderophore-independent Iron Transport System*

A TonB-independent ferric uptake system (Yfu) is present in pathogenic and nonpathogenic *Yersinia* spp. The Yfu system of *Y. enterocolitica* shows high AA identity (76.8–83.7%) to its *Y. pestis* homologue and also to the *Serratia marcescens* system (Saken et al., 2000; Gong et al., 2001). The *Y. enterocolitica* *yfu* locus is composed of four genes (*yfuA*, *yfuB*, *yfuC* and *yfuD*). A putative Fur binding site is found in the promoter region of *yfuA*. On the basis of similarities with other known proteins, YfuA (33.8 kDa in its mature form) is expected to be a periplasmic iron-binding protein, YfuB (56.5 kDa) is an inner-membrane permease containing a DNA binding site, YfuC (37.9 kDa) is probably an ATP-binding ABC transporter, and YfuD (22.8 kDa) is also probably an inner membrane permease (Saken et al., 2000). The function of the *Y. enterocolitica* Yfu system is supposed to be restricted, as is the function of the iron transport systems of other bacteria, to iron transport from the periplasm to the inner compartment of the cell and does not require a membrane receptor. Mutation in YfuB impairs neither the in vitro iron-utilization nor the virulence of *Y. enterocolitica* 1B/O:8 (Saken et al., 2000).

## Immunology (Ingo Autenrieth)

### Basic Considerations: Host Defense Against Extracellular and Intracellular Pathogens

As enteric *Yersinia* spp. are pathogenic for man as well as for rodents, data available on host responses against *Y. enterocolitica* and *Y.*

Table 36. The heme-uptake system of *Y. enterocolitica*.

Protein	Molecular mass (kDa)	Location	Putative function
HemP	6.5	UN	UN
HemR	78	Outer membrane	Heme receptor
HemS	39.2?	Cytoplasm	Heme-degrading enzyme
HemT	27	Periplasm	Heme-binding transport protein
HemU	35	Cytoplasmic membrane	Permease
HemV	31	Cytoplasmic membrane	Heme-specific ATPase

Abbreviation: UN, unknown.

*pseudotuberculosis* from both clinical studies in man and experimental animal infection models are considerable. Although experimental data from animals cannot be simply extrapolated to human yersiniosis, it is important to note that most, if not all, features of human yersiniosis (including enteritis and reactive arthritis) can be reproduced in various animal models including mice, rats and rabbits (Heesemann et al., 1993a). Therefore, it is conceivable that the features observed in these animal models may be relevant to the pathogenesis of human yersiniosis.

Immunity against enteric *Yersinia* spp. is based on innate and acquired host defense mechanisms. Most of the data available on this subject results from murine studies with *Y. enterocolitica*. Therefore, most of the data included herein may hold true for infections with *Y. enterocolitica* rather than for those caused by *Y. pseudotuberculosis*. This is important as *Y. enterocolitica* and *Y. pseudotuberculosis* may differ in their ability to induce immune responses. While *Y. enterocolitica* appears to be located exclusively extracellularly in host tissues, this is not yet completely established for *Y. pseudotuberculosis*. In fact, some of the data even imply that *Y. pseudotuberculosis* might be located both extra- and intracellularly in host tissues. This is an important basic consideration as immunity to microorganisms depends on their location in host tissues (Allen and Maizels, 1997; Autenrieth et al., 1999). Thus, extracellular pathogens are continuously exposed to components of the host defense such as complement or phagocytic cells as well as antibodies, while intracellular pathogens may evade these host defense systems. Therefore, it was believed that host responses and immunity to extracellular pathogens is rather trivial and a matter of polymorphonuclear leukocytes (PMNs), complement, and antibodies. However, expression of virulence factors by *Yersinia* spp. (e.g., Yops and YadA) enable *Yersinia* spp. to evade the innate host defense mechanisms including phagocytosis by PMNs and macrophages or complement attack. In consequence, mechanisms including helper (CD<sub>4</sub><sup>+</sup>) and cytotoxic (CD<sub>8</sub><sup>+</sup>) T cell responses, particularly  $\gamma$ -interferon-producing CD<sub>4</sub>T-helper 1 (Th1) cells, in cooperation with activated macrophages, are required to control *Yersinia* infection. Such host responses are considered as classical host defense mechanisms against (facultative) intracellular pathogens such as *Leishmania*, *Toxoplasma*, *Listeria*, *Mycobacteria* and *Salmonella* (Allen and Maizels, 1997). However, apart from *Yersinia*, other extracellular pathogens such as *Bacteroides* spp. or *Candida albicans* are also controlled by Th1-dependent host responses indicating that the Th1-Th2 paradigm may be of limited value. Moreover, recent work provided

further evidence that the classical segregation of host defense components appears to oversimplify their relevance to the control of intra- and extracellular pathogens, e.g., antibodies might also play a role in neutralizing intracellular bacteria and protecting against the intracellular pathogen *Listeria monocytogenes* (Edelson and Unanue, 2001).

### Host Defense Against *Yersinia* spp. at Mucosal Sites

Enteric *Yersinia* spp. are enteroinvasive pathogens. However, they cannot invade polarized intestinal epithelial cells from their apical side and therefore cannot simply invade the intestinal epithelial barrier (Coconnier et al., 1994; Schulte et al., 1998). Rather, they are taken up by M cells, which occur within the follicle-associated epithelial of the Peyer's patches (Grutzkau et al., 1990; Autenrieth et al., 1996b; F. Physiopathology). The M cells are specialized in that they take up particles from the intestinal lumen and transport these particles across the intestinal epithelium barrier. By this means, immune responses in the mucosa-associated lymphoid tissue are initiated, as the Peyer's patches are important components of the afferent limb of this system. Whether like in *Salmonella* infections (Vazquez-Torres et al., 1999) other cell types (e.g., CD<sub>18</sub>-expressing phagocytes or dendritic cells) are involved in invasion/uptake of *Yersinia* is unknown.

Recently, it was demonstrated in mice and human intestinal epithelial M-like cell lines that M cells, in contrast to enterocytes, expose  $\beta$ 1 integrins at their apical ("luminal") surface, which may bind to the *Yersinia* invasin protein (Clark et al., 1998; Schulte et al., 2000). This interaction leads to the transport of *Yersinia* across the epithelium. This strategy of entry is common in enteric pathogens including *Salmonella* spp. and *Shigella* spp. (Siebers and Finlay, 1996; Jepson and Clark, 1998). However, the invasion strategy of pathogens via M cells is a paradox, as antigen sampling by M cells is an important event for initiating mucosal immunity. In fact, the exploitation of M cells by pathogens stimulates the afferent mucosal immune system which is represented by the Peyer's patches. Thus, entry of enteric *Yersinia* spp. by this route is an ambiguous process. On the one hand, *Yersinia* may use the structure of the mucosal immune system for invasion of host tissue, and most likely for subsequent dissemination. On the other hand, the mucosal immune system is stimulated upon entry of *Yersinia*, and thereby host immune responses are triggered that, in most cases, will eliminate the pathogen.

One day after M cell invasion by *Y. enterocolitica*, small microabscesses consisting of PMNs

and extracellularly located *Yersinia* can be detected in Peyer's patch tissue (Hanski et al., 1989; Autenrieth et al., 1996b). In *Y. pseudotuberculosis*, granulomatous lesions can be observed, and the location (intra- versus extracellular) of *Y. pseudotuberculosis* is less clear (Physiopathology). The type of inflammatory responses induced in Peyer's patches can be considered as a net effect resulting from two contrary events: *Yersinia* spp. obviously trigger the recruitment of inflammatory cells such as PMNs which are normally absent from Peyer's patches. This could be a result of the function of *Yersinia* invasin protein. Recent work demonstrated that invasin, via binding to host cell  $\beta 1$  integrins, can trigger activation of proteins of the Rel family, e.g., nuclear factor kappa B (NF- $\kappa$ B) in epithelial cells (and macrophages) which leads to expression of various genes such as interleukin (IL)-8, granulocyte macrophage-colony stimulating factor (GM-CSF), and monocyte chemoattractant protein (MCP)-1 (Kampik et al., 2000; Schulte et al., 2000). These cytokines, particularly the chemokine IL-8, are proinflammatory cytokines and may cause the recruitment of PMNs. It is unclear whether and how other factors of *Yersinia* such as LPS (via binding to toll-like receptors [TLRs]) or YadA, Myf, etc. (which may interact with the surface of the host cells) contribute to the signaling for proinflammatory cytokine gene expression.

On the other hand, translocation of YopP/J via the type III secretion apparatus may suppress expression of proinflammatory genes via inhibition of NF- $\kappa$ B activation and may induce apoptosis in macrophages (Monack et al., 1997; Palmer et al., 1998; Ruckdeschel et al., 1998; Schesser et al., 1998). Recent work demonstrated direct evidence that YopP/J may account for such an effect in mesenteric lymph nodes and spleen in an experimental mouse infection model (Monack et al., 1998). This may also be important for a possible role of IL-1 in *Yersinia* infections. IL-1 production is also triggered by invasin-mediated NF- $\kappa$ B activation (Kampik et al., 2000). However, in contrast to IL-8, GM-CSF, or MCP-1, IL-1 is not released by the host cells. In *Shigella* infection, it was shown that IL-1 may be passively released by macrophages upon induction of apoptosis of the macrophages by *Shigella* (Sansone et al., 1995), and subsequently, IL-1 might augment the inflammatory host responses. It is conceivable that such a scenario might also occur in *Yersinia* infection.

Taken together, present data suggest that M cells and epithelial cells may be among the first cells encountered by yersiniae. Epithelial cells can produce inflammatory cytokines by which they signal to the host immune system the presence of pathogenic microorganisms. Therefore,

they have been called the "watchdogs for the natural immune system" (Eckmann et al., 1995). At present, it is not known whether M cells may also be capable of producing cytokines upon interaction with microorganisms. *Yersinia* might trigger the cytokine responses by binding to  $\beta 1$  integrins exposed at the basolateral surface of enterocytes. *Yersinia* may also elicit cytokine production causing proinflammatory cell reactions in the "pocket" adjacent to the basolateral side of M cells, where macrophages and lymphocytes are located.

Histological and immunohistological analyses suggest that innate host defense mechanisms including PMNs and macrophages are involved in control of *Yersinia* in Peyer's patches. Experimental approaches including the administration of neutralizing antibodies against adhesion molecules into mice prior to *Yersinia* infection showed that the adhesion molecules VLA-4 ( $\alpha 4\beta 1$  integrin, expressed on T cells, B cells, monocytes and involved in cell trafficking to sites of inflammation by interaction with VCAM-1 on endothelial cells) and Mac-1 (amb2 integrin, complement receptor 3 expressed on phagocytes and involved in binding and phagocytosis of pathogens and cell trafficking by binding to the ICAM-1 expressed on endothelial cells) are involved and play a protective role in the Peyer's patches (Autenrieth et al., 1996b). Thus, blocking of these host defense components by administration of specific antibodies to mice reduced phagocytosis of yersiniae and caused increased bacterial numbers in the tissue, which prevented clearance of *Y. enterocolitica* from Peyer's patches and led to fatal infection courses.

In addition the cytokines TNF- $\alpha$ , IL-12, IL-18 and IFN- $\gamma$  have been shown to be involved during mucosal infection (Autenrieth et al., 1996b; Bohn and Autenrieth, 1996; Bohn et al., 1998b; Hein, 2000). Thus, by means of neutralizing antibodies in mice deficient for cytokines or cytokine receptors, the protective role of these cytokines has been shown. However, it is not yet established to what extent and which of the cells in the Peyer's patches may produce these cytokines in the early phase of the *Yersinia* infection.

The role of complement in yersiniosis has been demonstrated by in vitro experiments. Thus, particularly YadA is involved in mediating resistance to complement and defensin lysis (Balligand et al., 1985; Pilz et al., 1992; Visser et al., 1996). As YadA is essential for survival of *Yersinia* in host tissue—and therefore is expressed in vivo (Autenrieth et al., 1996b)—it appears unlikely that the complement system, which obviously is subverted by virulence factors expressed by *Yersinia*, is actually involved in host defense against this pathogen. In keeping with this hypothesis, work in an experimental *Yersinia*

rat infection model showed that depleting rats had no effect on the bacterial numbers in infected organs, suggesting that the complement system has no protective role in *Yersinia* infections (Gaede et al., 1995b). Nevertheless, in the rat infection model, complement is important for reactive arthritis triggered by *Yersinia* spp. (Gaede and Heesemann, 1995a). *Yersinia*-triggered reactive arthritis occurs usually after resolution of the infection and is considered as the result of an immunopathological host reaction. Interestingly, in the experimental rat model of reactive arthritis, depleting rats by cobra venom factor suppressed *Yersinia*-induced reactive arthritis in Lewis rats. Whether this is due to reduction of the inflammatory reaction mediated by immune complexes composed of killed or degraded *Yersinia* precipitated in synovial tissue remains to be elucidated by further analysis.

Secretory IgA (sIgA) antibodies are secreted onto mucosal surfaces and may prevent invasion of pathogens. In fact, by means of a hybridoma tumor model, the transfer of hybridoma cells secreting monoclonal anti-*Salmonella* LPS antibodies prevented salmonellosis in mice (Michetti et al., 1992). In patients with yersiniosis, IgA antibodies directed against various antigens, including the Yop proteins, can usually be detected in serum, and in fact, is of considerable diagnostic value. The immunobiological role of sIgA antibodies in host defense against enteric *Yersinia* spp. is not yet established. Oral inoculation of recombinant *Y. enterocolitica* expressing a YopH-CtxB hybrid protein induced sIgA antibodies against the cholera toxin B subunit in the intestinal and respiratory tract and conferred protection of mice against a cholera toxin challenge (Van Damme et al., 1992). Moreover, preliminary work showed that sIgA is produced upon *Y. enterocolitica* O:8 wildtype infection in mice (C. Bielfeldt et al., unpublished observation). However, it is not clear whether sIgA antibodies might prevent invasion of the intestine by *Yersinia* or might contribute to control of the *Yersinia* or to modulation of the course of the infection. It might also be conceivable that the occurrence of a certain level of IgA antibodies in *Yersinia* infections might reflect an inappropriate T cell response including the development of strong Th2 responses resulting in chronic yersiniosis and possibly reactive arthritis rather than in control of the pathogen.

After replication in Peyer's patches, enteric *Yersinia* spp. disseminate via the lymphatics and possibly via the blood stream to the mesenteric lymph nodes, spleen, liver, lungs and peripheral lymph nodes (Physiopathology). The typical tropism for these organs is unclear and may reflect the tropism via adherence or invasion of *Yersinia*

for certain types of host cells or tissues. Preliminary data suggest that phagocytes expressing the Mac-1 molecule may be involved in the dissemination process of *Yersinia* (Autenrieth et al., 1996b). Likewise, in *Salmonella* infection CD18<sup>+</sup> host cells, possibly dendritic cells, have been demonstrated to be involved in intestinal translocation and dissemination as well as in induction of immune responses (Vazquez-Torres et al., 1999). Recent work demonstrated that *Y. enterocolitica* can be internalized by dendritic cells (DCs) and does not induce necrosis or apoptosis in these cells, but affects maturation of DCs (Schoppet et al., 2000). In fact, upon interaction with DCs, *Yersinia* modulates dendritic cell function including upregulation of CD83 and CD86 and a transient downregulation of MHC class II molecule expression resulting in decreased ability of dendritic cells to promote T cell proliferation (Schoppet et al., 2000). As T cell responses are crucial for resolution of *Yersinia* infection, modulation of DC functions might be a strategy to evade acquired immune responses by *Y. enterocolitica*. The role of DCs in yersiniosis should be further defined inasmuch as DCs are important sensors of infection and central in the development of immune responses against pathogens (Reis e Sousa, 2001).

#### Acquired Immunity to *Yersinia* spp.

Almost ten years ago, first direct evidence for a role of T cells in control of infection with enteric *Yersinia* spp. was reported (Autenrieth et al., 1992b). Today it is well established that *Yersinia* infection leads to strong T cell responses including CD4 and CD8 T cells, and that the T cells are involved in control of *Yersinia* (Autenrieth et al., 1992b; Autenrieth et al., 1993; Bohn and Autenrieth, 1996; Noll and Autenrieth, 1996; Bohn et al., 1998a). In fact, mice deficient in T cells are unable to control the pathogen and therefore develop chronic progressive and fatal infection. Consistently, the adoptive transfer of *Yersinia*-specific CD4<sup>+</sup> or CD8<sup>+</sup> T cells mediates resistance against a normally lethal challenge of *Yersinia*. As the protective CD4 or CD8 T cells produced cytokines such as IFN- $\gamma$  and IL-2, but not IL-4 or IL-10, it can be concluded that Th1 or TC1 cells (cytotoxic T cells that secrete  $\gamma$ -interferon) are protective in yersiniosis.

Th1 cells produce predominantly IFN- $\gamma$ , and it is established that IFN- $\gamma$  may activate macrophages, which in turn might be able to kill a pathogen. In fact, T cell-activated macrophages are likely to be the most important effector cells in yersiniosis. Interestingly, in these cells, *Yersinia* may induce apoptosis via translocation of YopP/J (Monack et al., 1997; Palmer et al., 1998; Ruckdeschel et al., 1998; Schesser et al., 1998).

Therefore, we can speculate that enteric *Yersinia* spp. can evade innate immunity including phagocytosis by PMNs and macrophages and possibly also, at least to some extent, acquired immunity in which macrophages are important mediators of effector functions as well as required for antigen presentation. By translocation of YopH into the cytosol of T cells or B cells, *Yersinia* may furthermore impair calcium fluxes in these cells and their ability to be activated through their antigen receptors (Yao et al., 1999). Thus, *Yersinia* may impair the development of acquired immunity. Nevertheless, as *Yersinia* infection is usually a self-limited disease, these immune evasion strategies are only partially successful, and at the end, *Yersinia* are obviously not capable of efficiently evading acquired immunity including phagocytosis and killing by most likely T cell-activated macrophages.

The role of cytokines in yersiniosis has been extensively studied in mice by comparison of mouse strains that are relatively susceptible (e.g., BALB/c) or resistant (e.g., C57BL/6) to *Yersinia* infection. One reason for this differential susceptibility of mice is their different ability to mount Th1 responses upon *Yersinia* infection including production of IFN- $\gamma$  (Autenrieth et al., 1994a; Bohn et al., 1994). *Yersinia*-resistant C57BL/6 mice can be rendered *Yersinia*-susceptible by in vivo neutralizing IFN- $\gamma$  with monoclonal antibodies (Autenrieth et al., 1994a). Vice versa, *Yersinia*-susceptible BALB/c mice can be rendered resistant by treatment with IFN- $\gamma$ . In keeping with these results, it was found that administration of neutralizing anti-IL-4 antibodies rendered BALB/c mice resistant against *Yersinia* infection. More recent work with cytokine- or cytokine receptor-deficient mice clearly demonstrated that the cytokines TNF- $\alpha$ , IL-12, IL-18 and IFN- $\gamma$ , and the transcription factor interferon consensus-sequence-binding protein are all essential for control of *Yersinia* infection (Bohn and Autenrieth, 1996; Bohn et al., 1998b; Hein et al., 2000). Although recent work suggested that in a very particular experimental setting including very high numbers of *Y. enterocolitica* in intravenous infections, 55-kDa tumor necrosis factor receptor (TNFRp55)-mediated mechanisms might be eventually detrimental to the host (Zhao et al., 2000). TNFRp55-dependent mechanisms in general are crucial for overcoming yersiniosis (Autenrieth and Heesemann, 1992a; Autenrieth et al., 1996b; Bohn et al., 1998b).

Epitope mapping of murine protective *Yersinia*-reactive T cell clones showed that a heat shock protein (HSP)60 of *Y. enterocolitica* is an immunodominant and protective antigen for CD<sub>4</sub> T cells and presented by MHC class II molecules (Noll et al., 1994; Noll and Autenrieth,

1996). Interestingly, the epitopes recognized by the T cells do not share significant homology to the corresponding mammalian HSP60 sequences. Recently, studies in humans confirmed these observations and demonstrated that *Yersinia* HSP60 is an immunodominant antigen for CD<sub>4</sub> and CD<sub>8</sub> T cells in reactive arthritis in humans (Mertz et al., 2000). A 12-mer core epitope (amino acids [aa] 322–333) of aa 319–342 peptide was recognized by T cell clones from one patient in the context of different human leukocyte antigen (HLA)-DR alleles suggesting that these findings might be relevant to other human individuals. Interestingly, although IFN- $\gamma$  was the most abundant cytokine secreted by these human T cell clones, some clones also secreted considerable amounts of IL-10. As IL-10 is an inhibitory cytokine, *Yersinia*-HSP60-triggered IL-10 secretion might promote bacterial persistence in chronic infections. Like in mice, all T cell clones reactive with *Yersinia* HSP60 did not crossreact with human HSP60. These results argue against the hypothesis that T cell responses against shared epitopes of HSPs might be involved in the pathogenesis of reactive arthritis after *Yersinia* infection. Whether *Yersinia* heat shock proteins might be released and stimulate innate immune responses by engaging toll-like receptors (TLRs; Wagner, 2001) is unclear. In summary, we can conclude that CD<sub>4</sub> T cells are important and essential for mediating the cellular host mechanisms required for control of enteric *Yersinia* spp.

CD<sub>8</sub> T cell responses have also been observed in murine and human yersiniosis. Moreover, the adoptive transfer of *Yersinia*-reactive CD<sub>8</sub> T cell clones can mediate immunity against a lethal challenge with *Y. enterocolitica*. However, the actual extent by which CD<sub>8</sub> T cells are involved in control of *Yersinia* infection remains to be further defined. Thus, in vivo depletion studies suggest a minor role of CD<sub>8</sub> T cells as compared with CD<sub>4</sub> T cells. However, in susceptible individuals such as BALB/c mice, IL-12-activated CD<sub>8</sub> T cells in concert with natural killer (NK) cells appear to be essential for pathogen control (Bohn et al., 1998a). In a *Yersinia* rat infection model, Lewis rats mount a *Yersinia*-specific RT-1-restricted CTL response (Falgarone et al., 1999). In this study, *Yersinia* invasin protein was required to mediate binding to blast target cells. In turn, YopE appeared to be translocated and presented via MHC class I molecules to CD<sub>8</sub> T cells. Both YopD and YopB were necessary for this process. The fact that Yop proteins are translocated into the cytosol of host cells explains a possible pathway of MHC class I antigen presentation for antigens of a bacterial pathogen that is extracellularly located. On the other hand, however, it is surprising that host cells that are

affected by several Yop proteins may still be capable of antigen processing, presentation and T cell stimulation. This is an issue that requires further molecular studies. Although acquired immune mechanisms usually succeed in control of *Yersinia* infections, enteric *Yersinia* spp. might interfere with this system and at least partially evade acquired immune responses. By translocation of YopH into the cytosol of T cells or B cells, *Yersinia* may impair calcium fluxes and their ability to be activated through their antigen receptors (Yao et al., 1999). T cells are inhibited in their ability to produce cytokines, and B cells are inhibited in their ability to upregulate surface expression of B7.2, an important costimulatory molecule. In consequence, such processes should significantly disturb cell-mediated immunity against the pathogen. As *Yersinia* may bind to integrins of B and T cells via invasin protein (Ennis et al., 1993), it is conceivable that lymphocytes, and thus the acquired immune response, is actually a target of the pathogenicity strategy of yersiniae in vivo. Interestingly, it was recently reported that *Yersinia* can translocate heterologous proteins such MAGE-A1 or *L. monocytogenes* p60 as fused to aa 1–130 of YopE into antigen-presenting (including dendritic) cells, which results in the presentation of antigenic peptides via MHC class I molecules (Chaux et al., 1999; Duffour et al., 1999; Ruessmann et al., 2000). These findings suggest that attenuated *Yersinia* strains might be suitable as a life vaccine carrier to induce MHC class I-restricted CTL responses against heterologous antigens.

In contrast to T cell responses, antibodies with protective abilities recognize the outer-membrane protein YadA of *Y. enterocolitica* (Vogel et al., 1993). Anti-LPS antibodies appear not to mediate significant protection. This might be explained by the fact that invasin or YadA, the two major outer membrane proteins of *Yersinia* might “cover” LPS structures. Consistently, studies in mice demonstrated that immunization with avirulent or virulent *Yersinia* mutant strains can confer immunity to challenge with highly pathogenic yersiniae and that antibodies against YadA are crucial in this process. Immunity conferred by YadA-specific antibodies appears to be serotype-specific, as antibodies against YadA from *Y. enterocolitica* serotype O:8 mediate protection against challenge with *Y. enterocolitica* O:8 pO:8 but not against challenge with *Y. enterocolitica* O:8 pO:9 (Vogel et al., 1993). Immunization of mice with an *S. typhimurium* strain expressing the outer-membrane protein *Yersinia* invasin induces anti-invasin antibodies in serum and intestinal secretion giving rise to inhibition of intestinal translocation, but failed to prevent *Yersinia* dissemination from the gut lymphoid tissue (Simonet et al., 1994). Together,

these data suggest that possibly different antigens are involved in the protective cellular and humoral immune responses.

Superantigens are microbial compounds that bind to conserved parts of MHC class II molecules and the V<sub>B</sub> region of T cell receptors thereby triggering the activation of certain subtypes of T cells and antigen-presenting cells (Superantigen of *Y. pseudotuberculosis*). In *Y. pseudotuberculosis*, a mitogenic activity was found in the culture supernatants that exhibited superantigenic activity (Abe et al., 1993). The superantigen encoding gene, *ypmA*, was subsequently disrupted in *Yersinia* strains. By means of an experimental mouse infection model, it was observed that upon intravenous infection a *Y. pseudotuberculosis ypmA*-deficient mutant was not affected in its ability to colonize the spleen, liver or lungs (Carnoy et al., 2000). However, the survival rate of infected mice was increased, and thus the virulence of a *ypmA* mutant strain was significantly reduced suggesting that YpmA contributes to the virulence of *Y. pseudotuberculosis* (Carnoy et al., 2000). By contrast, *Yersinia ypmA* mutants did not show any specific phenotype upon intestinal infections (Carnoy et al., 2000). Unfortunately, it has not yet been investigated in this model whether the effect of YpmA on innate or acquired immunity is responsible for this effect, and the mechanism(s) by which YpmA induces a higher death rate remains to be elucidated. In contrast to this observation, in vitro studies provided evidence that YpmA may (indirectly) reduce active ion transport and permeability in human T84 epithelial cell monolayers (Donnelly et al., 1999). Moreover, colonic tissue from YpmA-treated mice displayed a diminished responsiveness to cAMP-mediated secretagogues and nerve stimulation (Donnelly et al., 1999). Together, these findings imply that YpmA may contribute to the enteric symptomatology upon *Y. pseudotuberculosis* infection. In conclusion, the significance of *Yersinia* superantigen for modulation of immunity is, therefore, not yet clear.

### Mechanisms Involved in *Yersinia*-triggered Reactive Arthritis

Reactive arthritis is a “sterile” joint inflammation caused by infection with various microbial pathogens including enteric *Yersinia* spp. (Clinical manifestations of *Y. enterocolitica* infections). Although it is established that frequency of reactive arthritis is about 20-fold higher in patients with HLA B27 than in patients with other HLA alleles, the pathogenesis of reactive arthritis is still unknown. It is unclear how HLA B27 is linked to reactive arthritis. Several possible mechanisms including molecular mimicry or

whether HLA B27 is related to susceptibility for *Yersinia* infections have been discussed. Persistence of the pathogens is considered as an important factor in reactive arthritis and may depend on the genetic background of the host, the type of immune response mounted by the host, as well as on the arthritogenic potential of a given *Yersinia* strain. Thus, both a certain type of immunodeficiency that allows the persistence of the pathogen in host tissue, particularly at mucosal sites, as well as the generation of a strong immunopathological inflammatory response, are crucial in this process. In patients with reactive arthritis, immune complexes were demonstrated in the synovial fluid. Whether microbial antigens reach the joints as free antigens or as part of immune complexes is unknown. Previous studies failed to detect *Yersinia* DNA and/or viable yersiniae in the joints of these patients. A recent study, however, demonstrated the presence of *Y. enterocolitica* 16S rRNA in the synovial fluid of a patient (Gaston et al., 1999). These observations are in keeping with results from an experimental rat infection model of *Yersinia*-triggered reactive arthritis indicating that the pathogenicity, and thus, arthritogenicity of *Yersinia* spp., as well as complement, are important cofactors of reactive arthritis.

To the study the pathogenesis of this disease, a rat model of *Yersinia*-triggered reactive arthritis was established in 1987 and turned out to be clinically relevant and interesting (Hill and Yu, 1987). There are rat strains (e.g., Lewis rats) that develop reactive arthritis, while others (e.g., Fischer rats) do not (Gaede et al., 1992). As both Lewis and Fischer rats exhibit the identical MHC class I allele (RT.1), it appears unlikely that the MHC allele is the only gene linked to arthritis susceptibility. Interestingly, however, HLA B27 transgenic rats exhibit higher susceptibility for *Yersinia* infections than control rats. While certain highly pathogenic *Yersinia* wildtype strains induce reactive arthritis in rats, *Yersinia* mutants deficient in YadA, YopH or yersiniabactin production or uptake are attenuated and can no longer induce reactive arthritis in rats (Gaede et al., 1995c). Thus, the higher the virulence potential of a given *Yersinia* strain, the higher its potential for triggering reactive arthritis (Gaede et al., 1992; Gaede and Heesemann, 1995a). Moreover, de complementation of rats suppressed reactive arthritis triggered by *Yersinia* as determined clinically by an arthritis index, as well as by histomorphological investigations suggesting that complement and possibly immune complexes are involved in the pathogenesis of reactive arthritis (Gaede et al., 1995c). Interestingly, de complementation of rats did not change the bacterial numbers in infected organs, suggesting that the complement system does not sig-

nificantly contribute to the host defense against *Yersinia* (Gaede et al., 1995b). In the rat model, T cells appear to play a minor role in reactive arthritis, as treatment of rats with anti- $\alpha$ - $\beta$  TcR antibodies modulated adjuvant arthritis, which is known to be T cell-mediated, but not *Yersinia*-triggered reactive arthritis (Gaede et al., 1995c). Preliminary data in this model indicate that during the very early phase of the infection, *Yersinia* bacteria can be detected and even grown in the joints of both rat strains, suggesting that during the early phase of *Yersinia* infection the bacteria actually reach the joints. In both rat strains, the bacteria are rapidly eliminated. After two weeks, however, Lewis rats develop reactive arthritis and no viable bacteria can be grown from the joints at this time point.

More recently, it was reported that U937 cell transfectants expressing various HLA B27 constructs or other HLA alleles were unaltered in their ability to internalize *Salmonella* and *Yersinia* (Virtala et al., 1997; Ortiz-Alvarez et al., 1998), but were impaired in their ability to eliminate *Salmonella enteritidis* (Virtala et al., 1997), suggesting that HLA B27 monocytes might contribute to persistence of microorganisms that trigger reactive arthritis. Unfortunately, the mechanisms that account for this impaired intracellular killing have not been elucidated. In contrast to this study, no effect of endogenous HLA B27 on the survival of *Yersinia* or *Salmonella* was found in primary fibroblast cells (Huppertz and Heesemann, 1996; Huppertz and Heesemann, 1997).

As HLA B27 is an MHC class I molecule, it is conceivable that MHC class I-restricted immune responses may play a role in reactive arthritis. Patients with *Yersinia*-triggered reactive arthritis were found to recognize several antigens of *Yersinia* on the humoral or cellular level: the 19-kDa  $\beta$ -urease subunit protein of *Y. enterocolitica* O:3 is recognized by IgG and IgA antibodies, as well as by synovial fluid mononuclear cells and correlates with a synovial cellular immune response (Appel et al., 1999). Although these studies did not elucidate the actual pathogenic role of a certain antigen, the antigens identified might be used in screening systems and thus might help to identify cases with *Yersinia*-triggered reactive arthritis. Others found a common intra-articular oligoclonal T cell expansion in patients with reactive arthritis, providing further evidence for a molecular mimicry phenomenon at the T cell level in which arthritogenic microbial peptides may be presented by HLA B27 molecules (Dulphy et al., 1999). On the other hand, recent work showed that enterobacterial infection may downregulate expression of MHC class I molecules, particularly in patients with HLA B27 (Kirveskari et al., 1999). Taken together, the

pathomechanism of reactive arthritis and the role of HLA B27 molecules in this process remains obscure. Moreover, the causative relationship between other autoimmune disease such as Graves' disease or Hashimoto's thyroiditis and *Yersinia* infections is still questionable and remains to be elucidated further (Arscott et al., 1992; Chatzipanagiotou et al., 2001).

## Physiopathology (Francoise Guinet)

### Introduction

While all *Y. pestis* and *Y. pseudotuberculosis* isolates are potentially pathogenic to humans, *Y. enterocolitica* is heterogeneous with respect to virulence. Pathogenic strains, which belong to biogroups 2–5 (generally of serotypes O:3, O:5,27 or O:9), possess an array of virulence factors, such as the pYV plasmid (The pYV virulence plasmid) and the chromosomally encoded Inv (Invasin), Ail and Myf (Other putative virulence factors) adhesins, which are not present in the innocuous strains of biotype 1A (Cornelis et al., 1987; Miller et al., 1989; Pierson and Falkow, 1990; Iriarte et al., 1993). Highly pathogenic *Y. enterocolitica* strains (biotype 1B, often associated with serotype O:8) harbor, in addition to the above-mentioned virulence factors, a pathogenicity island (High Pathogenicity Island) encoding proteins involved in iron uptake. These strains provoke lethal disseminated infections in mice (Carter, 1975) and cause more frequent extraintestinal infections in humans (Bottone, 1999).

### The Enteropathogenic *Yersinia* Journey in Mammalian Hosts

COLONIZATION OF THE DIGESTIVE TRACT *Yersinia enterocolitica* and *Y. pseudotuberculosis* are enteric pathogens that normally enter the host through the oral route, usually by ingestion of contaminated food or water (Epidemiology of *Y. enterocolitica*). Ingested bacteria journey through the stomach and part of the gut lumen. A series of clinical case reports of *Y. enterocolitica* septicemiae suggested that stomach acidity may play a role in controlling the severity of the infection (Foberg et al., 1986). In vitro experiments, however, demonstrated that enteropathogenic *Yersinia* can survive in an environment at pH lower than 2 for at least 90 min in the presence of urea (De Koning-Ward and Robins-Browne, 1995; Young et al., 1996; Riot et al., 1997). In these in vitro experiments, urea concentrations normally found in healthy human stomachs (1.4 M) were sufficient to confer acid

resistance to *Y. enterocolitica* but not to *Y. pseudotuberculosis*, which may explain the observed difference in ileum colonization at early time points after intragastric inoculation (De Koning-Ward and Robins-Browne, 1995; Riot et al., 1997).

In various animal models and in humans, *Yersinia* preferentially settle and proliferate in the terminal ileum and in the cecum (Carter, 1975; Pai et al., 1980; Robins-Browne et al., 1985b; Skurnik and Poikonen, 1986). One reason for this specificity is the accumulation at this locale of Peyer's patches, through which enteropathogenic *Yersinia* enter the digestive tract (see below).

Another factor accounting for this preferred localization might be variations in mucus secretion along the intestinal tract. It has been noted that maximal mucus secretion occurs in distal ileum and in proximal colon during experimental infections of rabbits with *Y. enterocolitica* (Pai et al., 1980). *Yersinia pseudotuberculosis* and pathogenic *Y. enterocolitica* strains are able to bind to intestinal mucus preparations (Mantle et al., 1989; Paerregaard et al., 1991), and *Y. enterocolitica* O:3 strains were shown to multiply at high rates within mucus but not in the intestinal content (Paerregaard et al., 1991). It is not clear, however, whether mucus acts as a host defense mechanism by entrapping the bacteria and expelling them into the gut lumen before they can contact the gut epithelium, or whether mucus serves as a niche where *Yersinia* concentrate before colonizing and penetrating the digestive epithelium. In favor of the second possibility, lack of *yadA* expression correlates with decreased binding to mucus (Mantle et al., 1989; Paerregaard et al., 1991) and with reduced Peyer's patch colonization beneath the epithelium (Marra and Isberg, 1997). On the other hand, penetration of wild-type *Y. enterocolitica* through rabbit ileal mucus reduces subsequent attachment to brush border membranes, to levels comparable to those of an isogenic plasmidless derivative (Paerregaard et al., 1991). Perhaps, the best evidence so far for mucus binding being advantageous to the bacteria is that, at least for one *Y. enterocolitica* strain, the affinity to mucins from various species (human, rabbit and rat) correlates with the virulence potentials toward those animal species (Mantle et al., 1989).

It is conceivable that sequential deployment of adhesin expression during *Yersinia*'s journey directs the bacteria to specific settling and progression sites within the host (Isberg, 1990a; Bottone, 1999). For instance, expression of *yadA* was found to occur as early as 1 h after oral inoculation of *Y. enterocolitica* O:3 in rats, when the microorganism is present in the terminal ileum (Skurnik and Poikonen, 1986). Under in



vitro conditions, the temperature of maximal expression of the *ail* and *yadA* genes is the host temperature, i.e., 37°C (Kapperud et al., 1985; Bottone, 1999), but the *inv* gene (Invasin) is maximally expressed at temperatures below 30°C, which is paradoxical given invasin's primary importance in Peyer's patch colonization (see below). However, in a low pH environment, as can be encountered in the gastrointestinal tract, *inv* expression at 37°C is increased to levels similar to that of bacteria grown at 23°C (Pepe et al., 1994).

**TRANSLOCATION THROUGH THE GUT EPITHELIUM**  
Confirming earlier observations (Carter, 1975; Falcao et al., 1984), studies by Hanski et al. (1989) established that Peyer's patch (PP) is the main portal of entry for pathogenic *Y. enterocolitica* into the gut tissue. After oral infection, mouse PPs were 1000 times more heavily colonized than the surrounding mucosa, so that between 19 and 63 h postinfection, the number of bacteria in a single PP was equivalent to that in the rest of the ileal mucosa. Peyer's patches are specialized areas of the digestive tract, consisting of lymphoid follicles overlaid by the follicle-associated epithelium (FAE; Immunology). At the edge of FAE are cells devoid of a regular brush border, the so-called "M cells," which sample antigens from the intestinal lumen and translocate them to lymphocytes and macrophages residing in the basolateral pocket of these cells. Like other enteroinvasive pathogens, *Y. pseudotuberculosis* (Marra and Isberg, 1997; Clark et al., 1998) and pathogenic *Y. enterocolitica* species (Hanski et al., 1989; Grutzkau et al., 1990; Autenrieth and Firsching, 1996a) have been shown to utilize M cells to traverse the epithelial barrier. The *Yersinia* adhesin Inv binds to  $\beta$ 1-integrins apically expressed at the surface of M cells, thereby promoting internalization of the bacteria and its delivery to the basolateral side of the cell (Clark et al., 1998; Schulte et al., 2000). The Inv/ $\beta$ 1-integrin pathway is not the only way for enteropathogenic yersiniae to reach PPs in vivo, as an *inv yadA Y. pseudotuberculosis* double mutant was found to colonize PPs at early time points (90 min) even more efficiently than the wildtype (Marra and Isberg, 1997). However, the double mutation does not impart increased mouse virulence to *Y. pseudotuberculosis* (Han and Miller, 1997), contrary to an earlier report (Rosqvist et al., 1988). Also, *inv* mutants that express *YadA* appear to be trapped in the mucus layer lining the gut (Marra and Isberg, 1997), and they poorly colonize PPs as assessed by bacterial counts at 90 min (Marra and Isberg, 1997), 6 h and 18 h (Pepe and Miller, 1993). Therefore, the  $\beta$ 1-integrin/Inv interaction appears to be critical for liberating the bacteria from the mucus layer

to which they attach through their *YadA* fibrillae. The nature of the alternative ligand receptor pair permitting translocation through the digestive epithelium in the absence of Inv and *YadA* is not known, although it is likely that passage through M cells, here again, accounts for the specific targeting to PPs. Nonpathogenic *Y. enterocolitica*, which are devoid of the Inv, Ail and *YadA* adhesins, are rapidly eliminated from the host and display reduced or no gut wall invasive potential (Une, 1977a; Robins-Browne et al., 1985b).

Yersiniae are internalized almost exclusively by M cells, both in ligated-loop experiments (Autenrieth and Firsching, 1996a; Marra and Isberg, 1997) and in vitro (Schulte et al., 2000), which is in agreement with the fact that M cells, but not polarized enterocytes, express  $\beta$ 1-integrins on their apical side (Beaulieu, 1992; Clark et al., 1998; Schulte et al., 2000). However, it was recently shown in two epithelial cell lines that  $\beta$ 1-integrins exposed on the apical side of the tight junctions allowed for Inv-mediated binding of luminal *Y. pseudotuberculosis* and breaching of the epithelium through the cytotoxic effect of YopE (Tafazoli et al., 2000). The relevance of this finding in vivo remains to be elucidated.

**ESTABLISHMENT WITHIN PPs** After crossing M cells, pathogenic yersiniae proliferate locally within the PPs where they have been described, in various animal models, as extracellular colonies surrounded by phagocytes that display little or no phagocytic activity (Une, 1977b; Robins-Browne et al., 1985b; Lian et al., 1987; Hanski et al., 1989; Simonet et al., 1990; Grutzkau et al., 1993; Autenrieth and Firsching, 1996a). Phagocytosis inhibition is due to the activation of the pYV virulence plasmid harbored by pathogenic *Yersinia* strains (The pYV virulence plasmid). Strains cured of the pYV plasmid appear to be able to cross the digestive epithelium, but they are then rapidly eliminated from the host (Robins-Browne et al., 1985b; Skurnik and Poikonen, 1986; Lian et al., 1987; Grutzkau et al., 1993). Intraphagocytic pYV<sup>+</sup> yersiniae have been reported, but their viability has usually not been assessed (Skurnik and Poikonen, 1986; Lian et al., 1987; Pepe et al., 1995; Nikolova et al., 1997). One exception is the report of some actively dividing bacteria within phagocytes of piglets that had been infected per os, 1–6 days earlier, with a 4/O:3 *Y. enterocolitica* strain (Robins-Browne et al., 1985b).

While *YadA* has an ambiguous effect at the initial step of translocation across the epithelium (Paerregaard et al., 1991; Marra and Isberg, 1997), it is clearly necessary for *Y. enterocolitica* maintenance in mouse PPs (Pepe et al., 1995). This role is likely to be of critical importance for pathogenicity, as *Y. enterocolitica yadA* mutants

are totally avirulent (Pepe et al., 1995). For unclear reasons, *YadA* does not play the same role in *Y. pseudotuberculosis*, since *yadA* inactivation does not reduce virulence in this species (Bölin and Wolf-Watz, 1984; Han and Miller, 1997) nor ileum colonization (Kapperud et al., 1987b).

It is possible that binding of pathogenic yersiniae to extracellular matrix components is part of the gut wall colonization process. Collagen and laminin inhibit binding of various *Y. enterocolitica* strains to human intestine wall (Skurnik et al., 1994). *Yersinia enterocolitica* and *Y. pseudotuberculosis* *YadA* expression confers specific binding of *E. coli* constructs to purified type I, II, and IV collagen (Emody et al., 1989) and to fibronectin-coated coverslips (Tertti et al., 1992). The collagen binding properties are different for *Y. enterocolitica* and *Y. pseudotuberculosis* because the pYV plasmid is necessary for binding to type I, II and IV collagen in the former species, and only for binding to type I and type II collagen in the latter (Emody et al., 1989).

The extracellular PP environment is rich in serum proteins, including complement, which diffuse from blood vessels. Pathogenic yersiniae grown at 37°C manifest a high degree of resistance to the bactericidal action of serum from various species, including humans (Une and Brubaker, 1984). Complement depletion in mice by injection of cobra venom factor confirmed that, in mice, complement is not essential for the elimination of *Y. enterocolitica* (Hanski et al., 1991). Plasmid-encoded *YadA* and chromosome-encoded *Ail* have been implied in resistance to serum (Balligand et al., 1985; Tertti et al., 1987; Bliska and Falkow, 1992; Pierson and Falkow, 1993; Wachtel and Miller, 1995), although other studies failed to find a role of the *Y. enterocolitica* virulence plasmid in resistance to serum (Hanski et al., 1991) or in *Y. pseudotuberculosis* (Perry and Brubaker, 1983). Hence, resistance to serum is probably essential for yersiniae to settle within PPs. It might also be important for further spreading out through lymphatic or blood vessels, although intracellular spreading cannot be ruled out (see below).

**FROM PPs TO OTHER ORGANS** In the clinical setting, systemic infections by enteropathogenic yersiniae occur mostly in patients with underlying diseases and immunosuppressed conditions. Particularly prone are those patients with iron overload or treated with the iron-chelating agent desferrioxamine (Carniel, 1999; Other virulence factors, 3. Iron uptake systems). In previously healthy patients, the disease is usually limited to the gut wall or regional lymph nodes, although enteropathogenic yersiniae harboring the yersiniabactin-encoding high pathogenicity island

(i.e., *Y. pseudotuberculosis* I and III and *Y. enterocolitica* 1B) manifest a higher capability to spread in vivo and cause systemic infections (Bottone, 1999; Carniel, 1999; High Pathogenicity Island).

The likely route of emigration to mesenteric lymph nodes is via lymphatic vessels, whose function is indeed to drain tissue antigens toward lymph nodes. In rabbits, *Y. enterocolitica* organisms were seen in dilated lymphatic vessels of the lamina propria within 4 h after per os inoculation (Lian et al., 1987). After massive per os infection of mice with a highly virulent O:8 strain, bacteria were also occasionally seen in blood vessels at early times (3 h) after infection, thus probably accounting for the observed dissemination to other organs such as liver and spleen (Autenrieth and Firsching, 1996a). Notably, studies by Pepe and Miller uncovered an alternative route, bypassing PPs, for dissemination of *Y. enterocolitica inv* mutants from mouse gut lumen to liver and spleen (Pepe and Miller, 1993). *Yersinia enterocolitica* and *Y. pseudotuberculosis inv* mutants are as virulent in mice as their wild-type counterparts (Pepe and Miller, 1993; Han and Miller, 1997). In the *Salmonella* genus, it was recently shown that mutants deficient in invasion genes can bypass PPs and be directly transported from the gastrointestinal tract to the bloodstream inside CD<sub>18</sub>-expressing phagocytes (Vazquez-Torres et al., 1999). Although enteropathogenic yersiniae, unlike *Salmonella*, are mostly extracellular in PPs (Hanski et al., 1989), liver and spleen (Simonet et al., 1990), there are some lines of evidence suggesting that intracellular spreading might be utilized by yersiniae as well. Actively replicating pathogenic *Y. enterocolitica* were occasionally seen within phagocytes of animal gut walls after oral infections (Robins-Browne et al., 1985b). A recent report shows that *Y. pseudotuberculosis* can survive and replicate in macrophages in vitro by blocking phagosome acidification (Tsukano et al., 1999), and human neutrophils have the ability to translocate viable *Y. enterocolitica* through endothelial monolayers (Russmann et al., 1996). Additionally, reduced dissemination to the spleen has been observed in mice pretreated with antibodies specific for the CD<sub>11b</sub>/CD<sub>18</sub> heterodimer, which is expressed on the phagocyte surface (Autenrieth et al., 1996b). Noting that the  $\alpha 4\beta 1$ -integrin, which is involved in the homing of T lymphocytes to PPs, is the invasin receptor found on human T cell lines, Isberg also suggested that invasin, by binding to circulating lymphocytes, could promote bacterial spread within the host (Isberg, 1990a; Invasin).

### Histopathology

**ANIMAL MODELS** Mice challenged orally with high bacterial loads (e.g.,  $5 \times 10^8$  bacteria) have

been widely used for histopathological studies of *Y. enterocolitica* infections. In this model, the highly pathogenic 1B/O:8 bioserotype causes disseminated infections (Carter, 1975; Falcao et al., 1984), while low-pathogenic strains (biotypes 2–5) can establish long-term intestinal infections involving the PPs (Falcao et al., 1984; Ruiz-Bravo et al., 1999). In mice inoculated per os with a O:8 *Y. enterocolitica* strain, alterations of the gut wall include extensive bacterial proliferation in the PP's lymphoid follicle, and a major neutrophil reaction that within a few days results in PP destruction and surface ulcerations (Hanski et al., 1989; Grutzkau et al., 1993; Autenrieth and Firsching, 1996a). Various other laboratory animals have been used, including rabbits, rats, pigs and gnotobiotic piglets (Une, 1977a; Une, 1977b; Pai et al., 1980; Robins-Browne et al., 1985b; Skurnik and Poikonen, 1986; Nikolova et al., 1997). *Yersinia enterocolitica* in rabbits exhibit a higher tendency than in mice to elicit granulomatous responses (Une, 1977a; Robins-Browne et al., 1985b).

#### IN HUMANS

*Yersinia enterocolitica* Enterocolitis is the most common clinical presentation of *Y. enterocolitica* infection (West, 1997; Clinical manifestations of *Y. enterocolitica* infections). The corresponding histopathological images are those of a diffuse inflammatory reaction involving a prominent neutrophil influx in the lamina propria, without visible bacteria. Foci of superficial mucosal ulcerations can be present. Pathogenic *Y. enterocolitica* strains produce a heat-stable enterotoxin (YST, later named "YSTa") that is toxic to infant mice and induces diarrhea in young rabbits (Delor and Cornelis, 1992; Other virulence factors, 1. Heat stable enterotoxins). The *yst* gene is present in pathogenic strains and absent among nonpathogenic strains (Huang et al., 1997). Transcription of *yst* is induced in experimental conditions (temperature, pH, and osmolarity) similar to those of the ileum lumen (Mikulskis et al., 1994). YSTa toxin is thus likely to be important for pathogenicity, in spite of some discrepancies between YSTa expression and occurrence of symptoms in humans and laboratory animals (Robins-Browne et al., 1985b; Cover and Aber, 1989; Morris et al., 1991). Recently, two additional enterotoxins were discovered, but their role in pathogenicity is not established (Nair and Takeda, 1998).

Infection by *Y. enterocolitica* sometimes results in terminal ileitis, which histologically displays features of an invasive infection accompanied by a nonspecific acute inflammatory reaction, fitting pictures observed in animal models. Histopathological alterations in the ter-

минаl ileum and proximal colon are mostly restricted to PPs. In the PP lymphoid follicles, colonies of bacteria are overlaid by microabscesses. The necrotizing processes eventually disorganize the lymphoid follicle and erupt at the surface, creating well-delimited ulcerations over the PP areas. Histiocytes and lymphocytes can be seen beneath the microabscesses and the bacteria. Mesenteric nodes are enlarged and exhibit a lymphocytic reaction with necrotic foci that often contain colonies of *Y. enterocolitica*.

*Yersinia pseudotuberculosis* Contrary to *Y. enterocolitica*, acute enterocolitis and terminal ileitis are infrequent during *Y. pseudotuberculosis* infections, which typically result in mesenteric adenitis without visible bacteria (Gaulier and Poulton, 1983; West, 1997). As a consequence, *Y. pseudotuberculosis* is more rarely isolated from stools than *Y. enterocolitica*. Eight out of 13 (61.5%) *Y. pseudotuberculosis* clinical isolates characterized at the French National Reference Laboratory for *Yersinia* in 2000 were isolated from blood or organs, as opposed to 18 out of 176 (10.2%) pathogenic *Y. enterocolitica* clinical strains. Another difference between the two genera is that clinical and histopathological features suggest an acute disease in the case of *Y. enterocolitica* infections, while *Y. pseudotuberculosis* infections seem to evolve on a more subacute basis (Mollaret and Destombes, 1960c; West, 1997). Characteristically associated with *Y. pseudotuberculosis* infections is a strong macrophage reaction in the paracortical area of mesenteric lymph nodes, sometimes in the form of epithelioid granulomas of the tuberculoid type. The species name of this bacterium was coined from these tuberculosis-like reactions. Microabscesses seem to appear secondarily in the center of the granulomas. A lymphoid reaction inside and outside the germinal centers can be present.

## Antibiotic Susceptibility, Treatment and Prevention (Jeannette Pham)

### Antibiotic Susceptibility

*YERSINIA PSEUDOTUBERCULOSIS* *Yersinia pseudotuberculosis* clinical isolates are susceptible to most commonly used antibiotics such as ampicillin, cefotaxime, doxycycline, chloramphenicol, cotrimoxazole, nitrofurantoin and the fluoroquinolones.  $\beta$ -Lactamase has not been detected in isolates of this species (Jupeau et al., 1982; Van Noyen et al., 1995). By contrast, more than 50% of strains are resistant to the polymyxins in general and to colistin (polymyxin E; Jupeau et al., 1982; Van Noyen et al., 1995). Resistance to tetracycline has also been reported in some strains

of *Y. pseudotuberculosis* isolated from animals and birds (Reece and Coloe, 1985; Kanazawa et al., 1987).

**YERSINIA ENTEROCOLITICA** With the exception of  $\beta$ -lactam antibiotics, clinical isolates of *Y. enterocolitica* show in vitro susceptibility to antibiotics (such as gentamicin, chloramphenicol, cotrimoxazole and the fluoroquinolones) commonly used in Gram-negative infections, although a low number of isolates are resistant to tetracycline (Raevuori et al., 1978; Jupeau et al., 1982; Stock and Wiedemann, 1999).

Two types of chromosomal  $\beta$ -lactamase were described in *Y. enterocolitica*, the noninducible broad spectrum  $\beta$ -lactamase enzyme A with an isoelectric point (pH) of 8.7 and the inducible cephalosporinase enzyme B with pH's of 5.3 and 5.7 (Cornelis et al., 1973; Cornelis and Abraham, 1975). Strains of serotypes O:1, O:2, O:3 and O:9 produced two types of  $\beta$ -lactamase, enzyme A and enzyme B, while strains of serotype O:5,27 produced only enzyme B (Matthew et al., 1977); clusters of susceptibility to  $\beta$ -lactam antibiotics corresponding to the major serotypes have been observed (Hornstein et al., 1985). Environmental strains of *Y. enterocolitica* belonging to biotype 1A produce the broad spectrum enzyme A and an enzyme "B-like," a range of cephalosporinases similar to enzyme B, but with a different pH ranging from 6.2 to 7.3 (Pham et al., 2000). It has been demonstrated that although the genes encoded for both types of  $\beta$ -lactamase A and B are present in all *Y. enterocolitica*, they are not always active (De la Prieta et al., 1995).

In vitro, *Y. enterocolitica* are uniformly susceptible to cefotaxime, ceftriaxone, ceftazidime, aztreonam, cefpirome, imipenem and meropenem (Preston et al., 1995; Stock and Wiedemann, 1999; Pham et al., 2000). The susceptibility pattern of ticarcillin, cefoxitin and amoxicillin/clavulanate is specific to each biotype or subtype and depends on the extent of elaboration or the absence of one of the two  $\beta$ -lactamases, enzyme A and enzyme B (Pham et al., 2000). Owing to the lack of expression of enzyme A, biotype 2 (serotype O:5,27) strains are susceptible to ticarcillin (Pham et al., 1999). On the other hand, a number of *Y. enterocolitica* 4/O:3 isolated in Canada and all 4/O:3 strains isolated in Australia and New Zealand are highly susceptible to amoxicillin/clavulanate due to the absence of the cephalosporinase enzyme B (Pham et al., 1995). The borderline susceptibility to ampicillin often reported with strains of *Y. enterocolitica* biotype 1B, serotype O:8 originally isolated in North America is due to the presence of an enzyme "A-like" similar to enzyme A. This type  $\beta$ -lactamase has a slight shift in pH and is not as efficient in hydrolyzing ampicillin as

enzyme A expressed by strains of bioserotypes 2/O:9, 3/O:1,2a,3 and 4/O:3 (Pham et al., 2000).

## Treatment

**YERSINIA PSEUDOTUBERCULOSIS** Although, in vitro, *Y. pseudotuberculosis* are susceptible to all antibiotics commonly used, cefotaxime and especially ciprofloxacin are more efficient than cotrimoxazole and ampicillin in clinical situations (Jupeau et al., 1982; Nesbitt et al., 1994).

**YERSINIA ENTEROCOLITICA** Most patients with gastroenteritis recover without antibiotic treatment and antibiotic therapy. Although treatment shortens the intestinal carriage, it does not change significantly the duration of symptoms. First generation cephalosporins and amoxicillin/clavulanate are not effective when used alone in *Y. enterocolitica* septicemia (Gayraud et al., 1993), while intravenous cotrimoxazole, cefotaxime, ceftriaxone, alone or in combination with gentamicin, have been used successfully (Gayraud et al., 1993; Zhang et al., 1996a). Ciprofloxacin has been used with great success in *Y. enterocolitica* septic arthritis and osteomyelitis (Crowe et al., 1996).

## Vaccines

No vaccines are available for *Y. pseudotuberculosis* and *Y. enterocolitica* infections.

## Concluding Remarks (Elisabeth Carniel)

Since the emergence of *Y. pseudotuberculosis* and *Y. enterocolitica* as important human enteropathogens in the late 1960s, major advances have been made in the knowledge of the biology of these two species. However, what we currently know represents only the tip of the iceberg. One of the most promising tools for gaining a much deeper understanding of these bacteria is comparative genomics. The genomes of two strains of *Y. pestis*: ([http://www.sanger.ac.uk/Projects/Y\\_pestis/{CO92}](http://www.sanger.ac.uk/Projects/Y_pestis/{CO92})) (Sanger center) and (<http://www.genome.wisc.edu/html/pestis.html{KIM5}>) (University of Wisconsin) have been sequenced. The sequencing of the genomes of *Y. pseudotuberculosis* is close to being finished (Lawrence Livermore National Laboratory) (<http://www.greengenes.llnl.gov/bbrp/html/microbe.html{IP32953}>) and that of *Y. enterocolitica* ([http://www.sanger.ac.uk/Projects/Y\\_enterocolitica/{Ye8081}](http://www.sanger.ac.uk/Projects/Y_enterocolitica/{Ye8081})) is in progress (Sanger center). The comparison of these genomes, in association with functional studies, will most likely bring a tremendous amount of new information about the

biology of these species and the factors that define each species' specificities. The comparison with other Enterobacteriaceae genomes should also be extremely useful in understanding the evolution of the different members of this family.

## LINKS LPS

### Link 1: *Y. pseudotuberculosis* serotypes

The current *Y. pseudotuberculosis* antigenic scheme lists 15 O-serotypes (Tsubokura and Aleksic, 1995) of which the five first contain O-subtypes. The division into these types is based on ca. 30 O-factors (Table 29).

Differences in the first seven serotypes are in part determined by the presence of different DDHs (3,6-dideoxyhexoses) in the O-antigen. Many *Salmonella* and *E. coli* serotypes also contain DDHs in their LPS, which causes antigenic crossreactivity between them and *Y. pseudotuberculosis* serotypes (Tsubokura et al., 1984; Tsubokura et al., 1993). Epidemiologically, there are some differences in prevalence of the serotypes in human isolates. In Europe, the isolates belong most often to serotypes O:1–O:3 (Aleksic et al., 1995), while in Japan, they belong to serotypes O:4b, O:3, O:5a, and O:5b (Tsubokura et al., 1989). Apparently, the more recently recognized serotypes represent environmental *Y. pseudotuberculosis*, since they do not carry the virulence plasmid (Tsubokura and Aleksic, 1995; Nagano et al., 1997b).

### Link 2: Serotypes of *Y. enterocolitica* and related species

In *Y. enterocolitica* and related species, there are over 70 serotypes, which are mainly determined by the variability of the O-antigen (Wauters et al., 1991). Human and animal pathogenic strains of *Y. enterocolitica* that carry the virulence plasmid pYV (70–75 kb virulence plasmid of *Yersinia*) belong to certain serotypes; in Scandinavia and Europe, Canada, Japan, and South Africa, to O:3 and O:9, and in the United States, to O:8. Less frequently encountered pathogenic serotypes are O:4,32, O:5,27, O:13a,18, and O:21 (Table 30).

*Yersinia enterocolitica* strains of certain serotypes do not carry the pYV (Table 30) and these are collectively referred to as nonpathogenic or environmental serotypes. However, strains belonging to these serotypes are often isolated from stool samples of healthy humans. The virulence of different pathogenic serotypes varies, i.e., serotype O:8 strains are more virulent than those of serotypes O:3 or O:9. This is most evident as mouse lethality. The O:8 strains kill mice,

while the others do not unless iron is made available to the bacteria by pretreatment of mice with desferrioxamine or iron (Robins-Browne and Prpic, 1985a). On the other hand, O:3 strains frequently cause reactive arthritis in humans, while O:8 do so extremely rarely. It is not clear if O-antigen has any role in determining the differences in the virulence between the serotypes or whether this is due to other factors.

Serotype O:9 O-antigen crossreacts with *Brucella abortus* O-antigen causing diagnostic problems in serological confirmation of *Brucella abortus* infections (Ahvonen et al., 1969; Kittelberger et al., 1995a; Kittelberger et al., 1995b).

### Link 3: LPS biosynthesis

Polysaccharide biosynthesis precursors.

In Nature, biosynthesis of any kind of oligo- or polysaccharides involves NDP (nucleoside diphosphate)-activated sugars that function as sugar donors and corresponding glycosyltransferases that catalyze the transfer of the sugar moiety from NDP to the acceptor structure. Each sugar, acceptor structure, and specific glycosidic linkage between the sugar and acceptor structure needs its own specific glycosyltransferase.

LPS biosynthesis starts by the activation of sugar-1-P (phosphate), such as Glc (glucose)-1-P or Fru (fructose)-1-P by reaction with NTP (nucleoside triphosphate) to form NDP-activated sugars. The activation reactions take place in the cytoplasm, catalyzed by soluble enzymes. Different NDP-sugars are then synthesized in several enzymatic steps involving epimerases, reductases, hydratases, etc. Different NTP are used to activate different sugars, e.g., GTP for Man (D-mannose) and Rha (D-rhamnose), CTP for Abe (D-abequose) and Par (D paratose) UTP for Gal (D-galactose) and Glc (D-glucose), and dTTP for L-Rha; the reason for the choice is unclear. A few NDP-sugars needed for LPS biosynthesis are present in bacteria as intermediates of general metabolism; however, most of them have to be synthesized specifically for this purpose.

Lipid A – core biosynthesis.

Lipid A is assembled on the cytoplasmic face of the inner membrane (IM). In several enzymatic steps UDP-GlcNAc and fatty acids (supplied by acyl carrier protein) are converted into lipid A, which is a polar lipid with a backbone of GlcN-b-(1→6)-GlcN substituted with 4–7 N- and O-linked saturated fatty acid residues and 1–2 phosphate groups. The core oligosaccharide typically contains 10–15 sugar residues and is built on lipid A sequentially by specific glycosyltransferases that transfer the sugars from NDP-activated sugar precursors. The completed lipid

A-core molecules are translocated to the periplasmic face of the inner membrane (IM) where the O-antigen ligase, WaaL, ligates the O-specific polysaccharide (see below) to some of them. From the IM, the LPS molecules are finally translocated to the outer membrane (OM).

#### O-antigen biosynthesis.

The O-polysaccharide can be either homo- or heteropolymer (Fig. 38), i.e., a polymer of a single sugar or a polymer of repeats of different sugars (O-units), respectively, and their biosynthesis differs in some respects. Both types of O-polysaccharides have been found in the genus *Yersinia* (Link 4: *Y. enterocolitica* serotype O:3 LPS, Link 7: *Y. enterocolitica* serotype O:8 LPS).

Most steps in the O-antigen biosynthesis take place in the cytoplasmic face of the IM.

In the “heteropolymeric pathway,” each identical repeat unit is first assembled on a carrier lipid, Und-P (undecaprenol phosphate, a 55-carbon isoprenoid carrier lipid). Remarkably, the initiation reaction, transfer of the first sugar-1-P to Und-P, is in many strains accomplished by WecA, a GlcNAc-1-P transferase, the gene of which is located in the ECA (enterobacterial common antigen) gene cluster. In a few strains a dedicated transferase, such as a Gal-1-P transferase, is used for initiation. The O-unit assembly is then continued by dedicated glycosyltransferases that use NDP-activated sugar precursors. ‘Flippase’ protein, Wzx (Liu et al., 1996), translocates the completed Und-PP-O-unit to the periplasmic side of the IM. There, O-antigen polymerase, Wzy, polymerizes the O-units into long chains still carried by Und-P. The chain length determinant protein Wzz regulates the chain length. The polymerized chains are transferred from Und-P to the preformed lipid A-core structure by O-antigen ligase, WaaL.

In the “homopolymeric pathway,” the O-antigen polymer is synthesized on Und-P and completely elongated to full length in the cytoplasmic side of the IM by sequential transfer of sugar residues to the nonreducing end of the growing polysaccharide chain. The completed O-antigen is translocated to the periplasmic side by an ATP-driven transporter system, composed of Wzt and Wzm, that belongs to the ABC (ATP-binding cassette) transporter family (Whitfield, 1995). The O-antigen ligase, WaaL, transfers and ligates also the homopolymeric O-antigen onto lipid A-core.

**GENETICS** LPS biosynthesis requires enzymes that are specific for each LPS component and linkage, therefore the number of LPS genes is at least 50 and the genes are chromosomally located in most Gram-negative bacteria. The genes involved in lipid A biosynthesis are usually scattered around the genome, whereas those

involved in core biosynthesis form a distinct gene cluster as do the genes involved in O-antigen biosynthesis. In a few bacteria, LPS genes are found on plasmids or on temperate bacteriophage genomes.

#### Link 4: *Y. enterocolitica* serotype O:3 LPS

The lipid A structure is not known. The inner and outer core structures as well as that of the O-antigen are known (Radziejewska-Lebrecht et al., 1994; Radziejewska-Lebrecht et al., 1998; Table 31). Silver stained LPS from wildtype and outer core mutant strains of *Y. enterocolitica* O:3 are shown in Fig. 41, along with a schematic structure of the wild-type LPS.

The molecular genetic studies indicate that the outer core hexasaccharide of O:3 is an ancestral heteropolymeric O-unit (Skurnik et al., 1995). Furthermore, the structure of the *Y. enterocolitica* O:3 LPS is unique since the present O-antigen, which is a homopolymer of 6d-Alt (6-deoxy-L-altropyranose; Hoffman et al., 1980), is not attached to the outermost tip of the outer core but instead to the inner core (Skurnik et al., 1995). Similarly, in the enterobacterial common antigen lipopolysaccharide (ECA-LPS), the ECA moiety is ligated to the inner core (Radziejewska-Lebrecht et al., 1998).

#### Link 5: *Y. enterocolitica* serotype O:3 outer core

**Biosynthesis of the outer core.**

The biosynthesis of the outer core hexasaccharide (Fig. 42) needs 1) enzymes for the biosynthesis of NDP-sugar precursors, 2) six

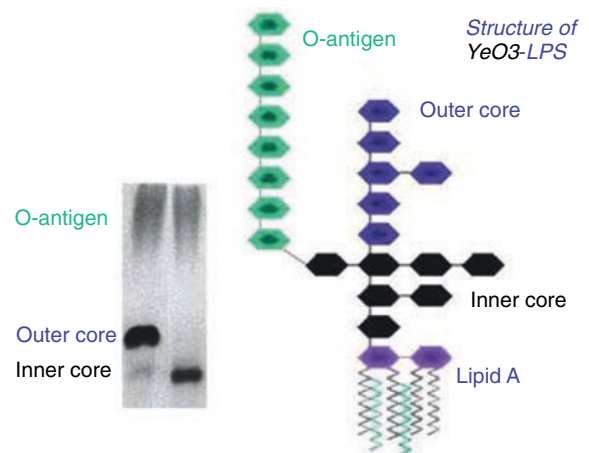
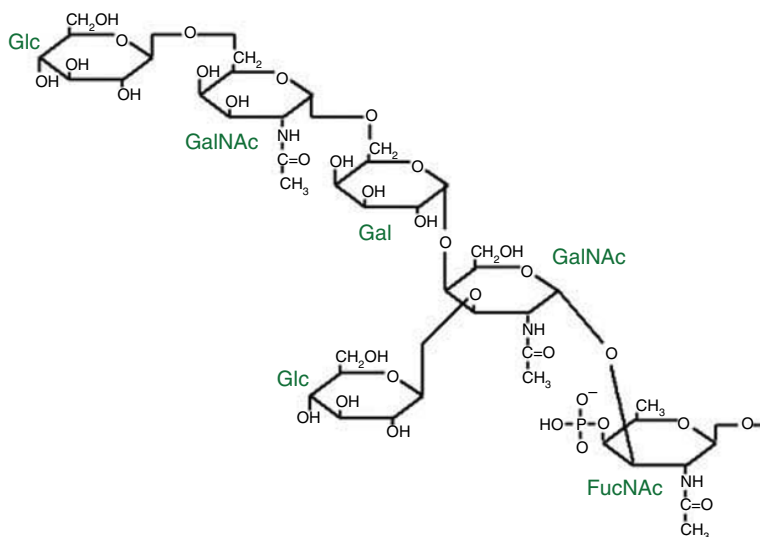


Fig. 41. *Y. enterocolitica* O:3 lipopolysaccharide (LPS). At left, silver stained deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) analysis of LPS: Left lane, LPS of wild-type, and right lane, LPS of mutant bacteria missing the outer core. At right, schematic structure of LPS.

Fig. 42. Outer core. Chemical structure of *Y. enterocolitica* O:3 outer core hexasaccharide.



glycosyltransferases, and 3) a flippase (Wzx). It is predicted that the biosynthesis of the hexasaccharide unit follows the heteropolymeric O-antigen biosynthetic scheme: it starts on the cytoplasmic face of the IM with the transfer of Fuc2NAc-1-P (*N*-acetylfucosamine or 2-acetamido-2,6-dideoxy-*L*-galactose) from UDP-Fuc2NAc to the carrier lipid Und-P (Skurnik, 1999a), and the rest of the hexasaccharide is then sequentially completed on Und-P-P-Fuc2NAc, where the coming sugar residues are transferred to the nonreducing end of the growing oligosaccharide. In the end, carbon 4 of the Fuc2NAc residue is decorated with a phosphate group (Fig. 42). After completion, Wzx flips the Und-P-P-hexasaccharide unit to the periplasmic face of the inner membrane (IM), where WaaL (the O-antigen ligase) ligates the hexasaccharide onto a lipid A-core structure.

The outer core gene cluster.

The outer core gene cluster (Skurnik et al., 1995; Table 32) contains nine genes (*wzx*, *wbcK*, *wbcL*, *wbcM*, *wbcN*, *wbcO*, *wbcP*, *wbcQ* and *gne*). Upstream of *wzx* is a noncoding region of 400 bp, preceded by the *hemH* gene. It is predicted that WbcK and WbcL are Glc-transferases, WbcM is a Gal-transferase, WbcN and WbcQ are GalNAc-transferases, and WbcO, a Fuc2NAc-transferase. WbcP is apparently involved in UDP-Fuc2NAc biosynthesis, and Gne is UDP-*N*-acetylglucosamine-4-epimerase (EC 5.1.3.7), thus providing UDP-GalNAc for outer core biosynthesis. Since UDP-Glc and UDP-Gal are products of general metabolism, the gene cluster appears to contain all necessary genes required for the outer core biosynthesis with the exception of the kinase to phosphorylate the C4-position of Fuc2NAc. The 39-bp reg-

ulatory JUMPstart sequence is present in the noncoding 400-bp region between *hemH* and *wzx* (Hobbs and Reeves, 1994).

The outer core is an ancestral O-unit?

The outer core gene cluster has a number of analogies with O-antigen gene clusters:

1. The flippase gene, *wzx*, is present in all known heteropolymeric O-antigen clusters but nowhere else (Klena and Schnaitman, 1993; Schnaitman and Klena, 1993; Liu et al., 1996; Reeves, 1994; Reeves et al., 1996).

2. In other *Yersinia*, the O-antigen gene clusters have the same location in the chromosome as the outer core gene cluster, i.e., between the *hemH* and *gsk* genes (Table 32).

3. The serotype O:3 homopolymeric O-antigen has a capsule-like structure and it is linked to the inner core (Skurnik et al., 1995).

4. In the O-antigen cluster of O:8, the O-antigen polymerase gene (*wzz*) is located between the *gne* and *gsk* genes (Zhang et al., 1996b; Zhang et al., 1997), but in the O:3 outer core cluster, there are no genes.

It can be speculated that the homopolymeric capsule-like O-antigen expressing gene cluster has taken over the O-antigen expressing role of the ancestral outer core gene cluster. The outer core biosynthesis in *Y. enterocolitica* O:3 was preserved since it is necessary for the integrity of the outer membrane and plays a role in virulence (Skurnik et al., 1999b).

Outer core is the bacteriophage  $\phi 37$  receptor and is present in other *Y. enterocolitica* serotypes (Table 29).

Phage 37 receptor is present not only in *Y. enterocolitica* serotype O:3 strains, but also in serotype O:1, O:2, O:5, O:5,27, O:6, O:6,31, O:9,

O:21, O:25,26,44, O:41(27)43, O:41,43, and O:50 strains, and in *Y. intermedia* O:52,54 strain (Skurnik et al., 1995). Phage resistant variants of some of these strains were analyzed by deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) and all had lost the outer core (Müller-Loennies et al., 1999; E. Ervelä and M. Skurnik, unpublished observation).

#### Link 6: *Y. enterocolitica* serotype O:3 O-antigen

The *Y. enterocolitica* O:3 O-antigen (Fig. 37) is a homopolymer of 6d-Alt (6-deoxy-L-altropyranose; Table 31) which is linked to the inner core of LPS (Hoffman et al., 1980; Skurnik et al., 1995). Genetic evidence indicates that the reducing end sugar is GlcNAc (see below). An O-antigen gene cluster, able to direct the synthesis of the homopolymeric O-antigen, must contain genes for 1) the biosynthesis of the NDP-activated sugar precursor, 2) at least two glycosyltransferases (one to initiate the O-antigen synthesis and the other to extend the homopolymer), and 3) a transporter system. Also, 6d-Alt is a carbon-3 epimer of L-Rha, and their biosynthetic pathways start similarly having dTDP-4-keto-6-deoxy-D-Glc as an intermediate. Biosynthesis of dTDP-L-Rha takes place in four steps, and the same is expected also for 6d-Alt. However, details of the dTDP-6d-Alt biosynthesis are not yet known and will need to be elucidated.

The O:3 O-antigen gene cluster (Table 32) contains 8 genes.

Upstream of the cluster are two ORFs of unknown function; there are no similar sequences in GenBank. The O-antigen cluster is organized into two operons and both operons are transcribed under tandem promoters; one is 1.2 kb upstream of *wbbS* and the other between *wbbU* and *wzm* (Zhang et al., 1993). The O-antigen expression is under temperature regulation; optimal expression takes place below 30°C. The first operon contains three genes *wbbS*, *wbbT* and *wbbU* and the second, five: *wzm*, *wzt*, *wbbV*, *wbbW* and *wbbX*, and all genes are essential for O-antigen synthesis (Al Hendy et al., 1991; Al Hendy et al., 1992; Zhang et al., 1993). There is a noncoding 1.5-kb region upstream of *wbbS* that contains relics of the *rmlB* gene and the regulatory JUMPstart sequence.

#### Biosynthesis of the O-antigen.

*WbbS*, *WbbV* and *WbbW* are significantly similar to *RmlC*, *RmlD*, and *RmlA*, respectively, the enzymes involved in the dTDP-L-Rha biosynthesis, indicating that they function in dTDP-6d-Alt biosynthesis. The initiation of the O-antigen biosynthesis is *WecA* (GlcNAc-transferase) dependent (Zhang et al., 1996b). Thus the O-antigen biosynthesis utilizes an initiation step

where a single GlcNAc residue is transferred to Und-P. After this, a specific 6d-Alt-transferase transfers the first 6d-Alt residue to GlcNAc-Und-PP, upon which the homopolymer is then sequentially built up by a second 6d-Alt-transferase that uses (6d-Alt)<sub>n</sub>-GlcNAc-Und-PP as an acceptor. Both *WbbT* and *WbbU* share conserved local motifs with a number of glycosyltransferases (Morona et al., 1995; Skurnik et al., 1995), and fulfill the need for two glycosyltransferases.

#### O-antigen transport.

*Wzm* and *Wzt* make up an ATP-driven polysaccharide transporter system that transports the Und-PP-bound O-antigen to the periplasmic space (Zhang et al., 1993; Whitfield, 1995). Transposon insertions into the *wzm* and *wzt* genes result in accumulation of cytoplasmic O-antigen (Zhang et al., 1993). *Yersinia enterocolitica* O:3 was the first organism where an ATP-driven polysaccharide transport system was recognized in an O-antigen gene cluster.

#### Serotype O:3 related serotypes.

6d-Alt is the major component of *Y. enterocolitica* O:1,2a,3 and O:2a,2b,3 O-antigens (Table 31). The genetic organization of the O-antigen gene clusters of these organisms is not known. The O:3 O-antigen cluster DNA hybridizes strongly with DNA from these organisms, indicating the presence of similar clusters (Al Hendy et al., 1991).

#### Link 7: *Y. enterocolitica* serotype O:8 LPS

##### Structure of *Y. enterocolitica* O:8 LPS

Silver stained deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) and a schematic structure of the LPS of *Y. enterocolitica* O:8 is shown on Fig. 43. Details of the chemical structure are given in Table 31. The inner core structure is very similar to that of O:3 and O:9. The O-unit is a branched pentasaccharide. Interestingly, the O-antigens of serotypes O:7,8 and O:19,8 are almost identical to that of O:8 (Table 31).

#### Link 8: *Y. enterocolitica* serotype O:8 O-antigen

##### Biosynthesis of O-antigen.

The O:8 O-unit is a branched pentasaccharide (Fig. 44). The gene cluster for the biosynthesis of the O-antigen should contain 1) biosynthetic genes for each individual NDP-sugar (except for UDP-Gal, the biosynthesis of which belongs to general metabolism), 2) genes for five glycosyltransferases, one for each residue, 3) the *wzx* gene for flippase needed in heteropolymeric O-antigen transport, 4) the *wzy* gene for O-antigen



polymerase, and 5) the *wzz* gene for the chain length determinant.

The *Y. enterocolitica* O:8 O-antigen gene cluster contains 18 genes, spanning about 19 kb of chromosomal DNA (Zhang et al., 1997). Nine genes encode enzymes for the biosynthesis of UDP-GalNAc (*gne*), GDP-Man (*manB* and *manC*), GDP-Fuc (*gmd* and *fcl*) and CDP-6d-Gul (*ddhA*, *ddhB*, *wbcA* and *wbcB*). Of the remaining nine genes five, *wbcC*, *wbcD*, *wbcG*, *wbcH* and *wbcI* code for glycosyltransferases, *wzx* codes for a flippase, *wzy* for an O-antigen polymerase, and *wzz* for a chain-length determi-

nant. One gene, *wbcF*, remains still unassigned (Zhang et al., 1997).

Link 9: *Y. pseudotuberculosis* LPS

No genetic work has been reported on the lipid A and core biosynthesis of *Y. pseudotuberculosis*. The core structure has been reported but still the details of the structure are missing (Ovodov et al., 1992). In silver-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis lipopolysaccharide (SDS-PAGE LPS) of most *Y. pseudotuberculosis*, serotypes shows the ladder-like staining pattern as an indication of heteropolymeric O-antigen. However, detection of the ladder of a few serotypes, e.g., O:1b, is very difficult by silver staining. This is due to absence of vicinal hydroxyl-groups in the chemical structure of the O-unit (a requirement for silver staining) and/or expression of very low amounts of the O-antigen by the strain.

*Y. pseudotuberculosis* O-ANTIGEN

The O-antigen structures of a number of *Y. pseudotuberculosis* serotypes are known (Table 31).

The chromosomal location of the O-antigen gene cluster in *Y. pseudotuberculosis* and also of the cryptic O-antigen gene cluster in *Y. pestis* is between the *hemH* and *gsk* genes (Skurnik et al., 2000). On the basis of sequencing, gene-specific PCR analysis, and hybridization studies, it has been established that all except serotypes O:7, O:9 and O:10 carry the DDH (3,6-dideoxyhexose) biosynthesis genes, named “*ddhDABC*,” or their homologues in the beginning of their gene clusters, downstream of the *hemH* gene and the JUMPstart sequence (a 39-bp element common to several polysaccharide gene clusters). The *ddh*

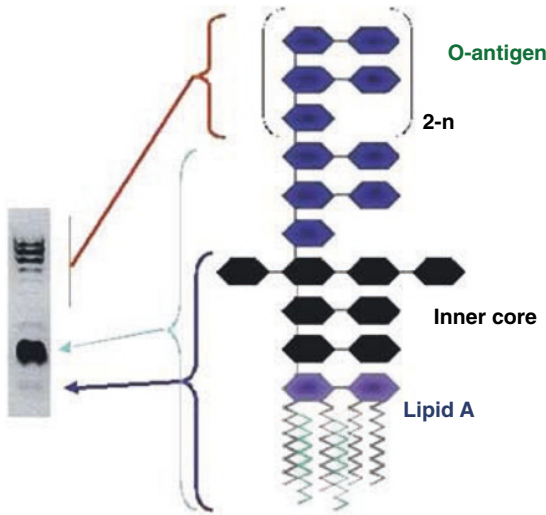


Fig. 43. *Y. enterocolitica* O:8 lipopolysaccharide (LPS). At left, silver stained deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) analysis of *Y. enterocolitica* O:8 LPS. At right, schematic structure of LPS. Different populations of molecules are indicated.

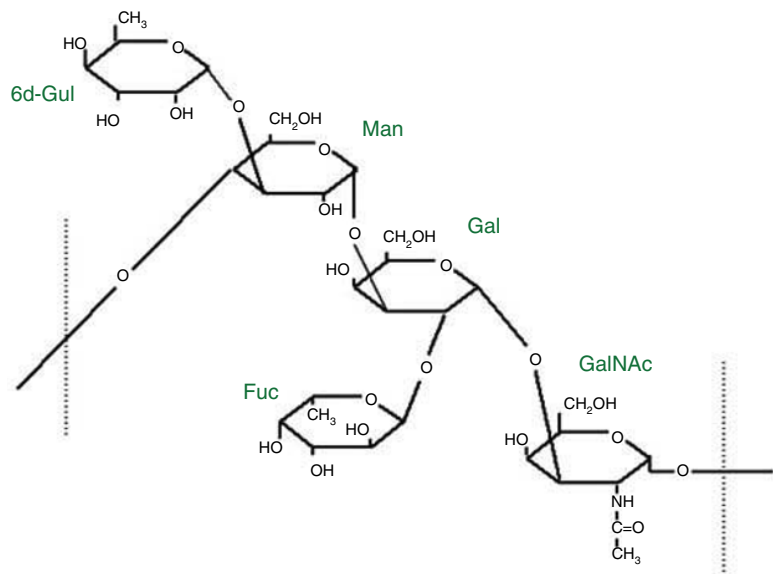


Fig. 44. O-antigen. Chemical structure of the repeat unit pentasaccharide of *Y. enterocolitica* O:8 heteropolymeric O-antigen.

genes are very similar to those of *Salmonella* (Kessler et al., 1993; Hobbs and Reeves, 1995). DDH reaches a biosynthetic intermediate with the help of the *ddhDABC* block encoded enzymes, and completion of the specific DDH in each serotype requires one or two additional genes that determine the serotype specificity, e.g., *abe* in serotypes O:2a, O:2b, and O:2c; *prt* and *tyv* in serotypes O:4a and O:4b; *prt* in serotypes O:1a and O:1b, and *ascEF* in serotype O:5a; *ascEF* genes are not present in *Salmonella* and are therefore *Y. pseudotuberculosis* O:5a specific (Thorson and Liu, 1993a; Thorson et al., 1993b; Thorson et al., 1993c; Thorson et al., 1994).

In addition to the DDH pathway genes, the O-antigen clusters contain the genes coding for the biosynthetic enzymes of the other sugar components, the respective glycosyltransferases, and the translocation and polymerization proteins Wzx, Wzy and Wzz. Interestingly, on the basis of PCR analysis, all serotypes appear to use a highly identical Wzz protein for chain length determination (Skurnik et al., 2000).

Comparative analysis of a number of *Y. pseudotuberculosis* O-antigen gene clusters just downstream of the *ddh* genes revealed footprints of homologous recombination events and relics of insertion sequence IS630 also observed in *Shigella sonnei*, relating to the evolution of the clusters (Hobbs and Reeves, 1995). Another characteristic feature of the O-antigenic clusters of *Y. pseudotuberculosis* is the presence of numerous intergenic 6–8 nucleotide repeats (Skurnik et al., 2000). CDP-DDH biosynthesis.

DDHs are formed via a complex biosynthetic pathway beginning with CDP-D-hexoses. Biosynthesis of CDP-Asc (ascarylose), one of the naturally occurring DDH, consists of five enzymatic steps, with CDP-6-deoxy-D-3,4-glucose reductase (E3), a homogeneous enzyme composed of a single polypeptide with a molecular weight of 39,000, participating as the key enzyme in this catalysis (Lo et al., 1994). The corresponding gene (*ascD=ddhD*) is located in the ascarylose biosynthetic cluster. E3 is an NADH (hydrogenated [reduced] nicotinamide-adenine dinucleotide)-dependent enzyme that catalyzes the key reduction of the C-3 deoxygenation step during the formation of CDP-Asc. One FAD (flavine-adenine dinucleotide) and one plant ferredoxin type iron-sulfur center were found to be associated with each molecule of E3 (Miller et al., 1993). E3 employs a short electron-transport chain composed of both FAD and the iron-sulfur center to shuttle electrons from NADH to its acceptor. Cys-75 and Cys-296 may be important for electron transfer between NADH, FAD, and the iron-sulfur center (Ploux et al., 1995).

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## *Yersinia pestis* and Bubonic Plague

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### Introduction

Caused by *Yersinia pestis*, bubonic plague (also known as “the pestilence” or “Black Death”) is the most devastating epidemic bacterial disease known to humankind. This appraisal is based upon the terrifying manifestations of plague (Butler, 1983; Pollitzer, 1954), which include death within 1–3 days following the onset of symptoms (Butler, 1983). Up to 200 million people have succumbed to this disease over the course of history (Duplais, 1988). On the other hand, the closely related *Yersinia pseudotuberculosis* or the more divergent *Yersinia enterocolitica* (termed “enteropathogenic” yersiniae because they typically produce gastrointestinal afflictions) do not have the ability of plague bacilli to cause comparable acute lethal disease in rodents and primates. Nevertheless, these three human parasites exhibit a similar basic pattern of pathogenicity mediated by a shared ~70-kb plasmid termed “pCD” in *Y. pestis*, “pYV,” “pCad” or “pIB” in *Y. pseudotuberculosis*, and “pYV” in *Y. enterocolitica* (Ben-Gurion and Shafferman, 1981; Cornelis and Wolf-Watz, 1997; Ferber and Brubaker, 1981; Goguen et al., 1984; Hu et al., 1998; Iriate and Cornelis, 1996; Perry and Fetherston, 1997; Perry et al., 1998). Hereafter, the term pCD will apply to the ~70-kb plasmid of *Y. pestis* and pYV will designate its analog in both *Y. pseudotuberculosis* and *Y. enterocolitica*. Cells of wild-type *Y. pestis*, most but not all serotypes of *Y. pseudotuberculosis*, and invasive isolates of *Y. enterocolitica* also possess an ~35-kb chromosomally encoded high-pathogenicity island (Bearden et al., 1997; Buchrieser et al., 1998a; Buchrieser et al., 1998b; Buchrieser et al., 1999; Carniel et al., 1996; Fetherston et al., 1995; Fetherston and Perry, 1994; Fetherston et al., 1992). The shared plasmid encodes a battery of cytotoxins termed “Yops” and at least three anti-inflammatory activities, whereas the high-pathogenicity island contains genes required for the siderophore (yersiniabactin)-dependent assimilation of iron. Superimposed on this common theme are two unique plasmids in *Y. pestis* that facilitate invasion of tissues and colonization of the flea vector. In addition, plague bacilli have

lost central enzymes of intermediary metabolism and host cell adhesin and invasins activities required by the enteropathogenic yersiniae to express chronic infection (Achtman et al., 1999; Brubaker, 1991; Brubaker, 2000; Hinnebusch, 1997). As a consequence of this genomic fine-tuning, plague has become the yardstick by which all other acute infectious diseases are measured. *Yersinia pestis* is now more firmly entrenched in endemic foci throughout the world than at any previous time (Baltazard, 1960; Perry and Fetherston, 1997). Although controllable by antibiotics, the emergence of drug-resistant isolates has been reported (Galimand et al., 1997). The probability of acquiring the disease from natural reservoirs (sylvatic plague) will increase as the human population continues to press into endemic regions. Finally, the possibility that *Y. pestis* will be utilized as an agent of terrorism is sobering. Accordingly, it is imperative to fully understand the molecular workings of this formidable pathogen so that alternative strategies to antibiotic therapy can be developed to combat the probable recrudescence of plague in humans.

Considerable progress toward a better understanding of the shared features of plague and the chronic diseases caused by the enteropathogenic yersiniae recently has been made. From the discovery of *Y. pestis* in Hong Kong by Alexander Yersin in 1896 until well into the 1980s, the symptoms of plague were generally viewed as the consequence of a relentless and uncompromising conflict between host and parasite (Brubaker, 1972; Burrows, 1963; Butler, 1983; Pollitzer, 1954). However, results of later experiments indicate that these symptoms more accurately reflect the combination of stealth and deception effects, which cause the host to ignore the mortal danger of ongoing systemic invasion. In this context, the “stealth” refers to the ability of docked yersiniae to translocate cytotoxins directly into host cell cytoplasm via a type III protein secretion system (Michiels et al., 1990), thereby effectively bypassing the immune system. Similarly, use of the word “deception” characterizes the capacity of *Y. pestis* to suppress inflammation by downregulating proinflammatory cytokines (Nakajima and Brubaker, 1993). Both of these

mechanisms, as well as ancillary activities required for growth *in vivo* and invasion of tissues, have undergone intensive recent study and are considered under Disease.

As already noted, an interesting feature of *Y. pestis* is its remarkably close homology to *Y. pseudotuberculosis* (Brenner et al., 1976; Moore and Brubaker, 1975), an organism capable of causing chronic disease in a variety of mammals and birds. *Yersinia pseudotuberculosis* has long been recognized as the immediate forebear of *Y. pestis*, and analysis of restriction enzyme fragment polymorphism place the occurrence of divergence just 1,500 to 20,000 years ago (Achtman et al., 1999). The close genetic relationship between these two species and the dramatic differences in symptoms of plague versus human pseudotuberculosis thus provide a unique model for defining the nature of acute disease. As might be expected, differences between the genomes of these yersiniae are modest; they comprise the acquisition by *Y. pestis* of two unique plasmids and loss of chromosomal genes that, in *Y. pseudotuberculosis*, facilitate expression of enteropathogenic disease and efficient intermediary metabolism. Nevertheless, these differences cause marked phenotypic distinctions, which are defined under Physiology. The putative sequence of events and selective pressures favoring the emergence of *Y. pestis* from *Y. pseudotuberculosis* are considered under Phylogeny.

Overviews of *Y. pestis* and its virulence factors are available (Brubaker, 1991; Brubaker, 2000; Perry and Fetherston, 1997) as are descriptions of the disease (Bahmanyar and Cavanaugh, 1976; Poland and Barnes, 1979; Pollitzer, 1954); the book by Thomas Butler provides an especially vivid picture of plague (Butler, 1983). The important role of the shared -70-kb plasmid with emphasis on the translocation of virulence effectors via a type III mechanism of protein secretion has been reviewed in detail (Cornelis, 1998a; Cornelis, 1994; Cornelis, 1992; Cornelis et al., 1998b; Cornelis and Wolf-Watz, 1997).

## Phylogeny

As noted in the Introduction, *Y. pestis* is a recently evolved clone of *Y. pseudotuberculosis* (Achtman et al., 1999). The close relationship between these species provides an excellent model for defining the nature of acute systemic disease (typified by plague) as opposed to the chronic and localized lymphadenitis and gastroenteritis usually caused by *Y. pseudotuberculosis* (Daniels, 1973). The remarkably small number of phenotypic differences known to exist between the two species (Table 1) has prompted speculation as to the nature of hereditary changes

required for expression of acute disease and the order of their occurrence (Achtman et al., 1999; Brubaker, 1991; Brubaker, 2000; Hinnebusch, 1997). Tacit to all models of emergence is recognition that cells of *Y. pestis* have both gained new genetic information not shared by *Y. pseudotuberculosis* and lost functions that its progenitor has retained.

Unique traits of *Y. pestis* known to mediate the acute symptoms of plague are defined under Disease; they consist of pPCP-encoded plasminogen activator (Pla) plus pMT-encoded capsular fraction 1 antigen (Caf1) and murine toxin. Chromosomally encoded virulence functions particular to *Y. pestis* have not been established as yet but certainly may exist. The present extrachromosomal existence of the structural genes encoding Pla, Caf1 and murine toxin strongly suggests their recent acquisition by lateral transfer, despite the fact that neither pPCP nor pMT exhibit sex-factor activity (Hu et al., 1998; Lindler et al., 1998). This deficiency is inconsequential in the case of pPCP, which is sufficiently small (9.7 kb) to readily co-conjugate with the sex factor F (Kutyrev et al., 1999). However, larger plasmids (such as pMT, 100.9 kb) might not be expected to undergo lateral transfer in nature via co-conjugation or transformation, although transfer by transformation can be accomplished in the laboratory. The *raison d'être* of plasmids is to provide the host bacterium with some novel or unique capability to facilitate survival in an otherwise hostile environment. The plasmid pMT is an enigma in this sense in that it encodes not only structural genes for Caf1 and murine toxin (thereby providing the requisite selective advantage), but also genes for a number of plebeian functions probably concerned with normal vegetative growth (Hu et al., 1998; Lindler et al., 1998). Two additional findings emphasize this conundrum. First, pMT, in its entirety, can readily integrate into the chromosome (Protsenko et al., 1991). Second, the -18-kb operon of pMT encoding the structural gene for Caf1 (*caf1*) and attendant functions is linked to bacteriophage homologs and possesses a distinct guanine plus cytosine (G+C) content; pMT may thus exist as a second high-pathogenicity island (Hu et al., 1998; Lindler et al., 1998). These observations are consistent with the notion that the genes encoding Caf1 and murine toxin may now reside in locations different from those first entered in the genome.

In accord with the prospect of a flexible genome is the important discovery by Portnoy and Falkow (1981a) that up to 30 copies of the insertion sequence (IS) element IS100 exist within the chromosome of *Y. pestis*, as well as at least one copy within each plasmid. This element was sequenced (Podladchikova et al., 1994) and

Table 1. Phenotypic distinctions between typical strains of *Y. pestis* and *Y. pseudotuberculosis*.

Determinant	<i>Y. pestis</i>	<i>Y. pseudotuberculosis</i>	Mutational event	Consequence in <i>Y. pestis</i> <sup>a</sup>	References
pPCP	+	0	Lateral transfer	Acquisition of plasminogen activator (accelerated dissemination in host tissues and posttranslational degradation of undelivered Yops) Acquisition of pesticin (assures retention of pPCP)	Ben-Gurion and Shafferman, 1981 Ferber and Brubaker, 1981 Protsenko et al., 1983
pMT	+	0	Lateral transfer	Acquisition of capsular fraction 1 antigen (resistance to phagocytosis) Acquisition of murine toxin (survival in the flea vector and prompt death of murine hosts)	Kutyrev et al., 1986 Protsenko et al., 1983 Protsenko et al., 1991
Pgm <sup>+</sup>	+	0	Possible constitutive expression of <i>hmsT</i>	Ability to block flea proventriculus	Hare et al., 1999 Jones et al., 1999
Lipopolysaccharide O group structure at 26°C	0	+	Cryptic R genes	Constitutive resistance to complement (alternative pathway)	Federova and Devdariani, 1998 Minka and Bruneteau, 1998 Yersinia pestis Sequencing Group Rosqvist et al., 1988
YadA	0	+	Frameshift	Minimized association with host cell surfaces	Simonet et al., 1996
Inv	0	+	IS1541 insert	Minimized association with host cell surfaces	Torosian and Zsigray, 1966
Ail	0	+	IS285 insert	Minimized association with host cell surfaces	
Flagella at 26°C	0	+	Cryptic H genes	Elimination of unnecessary function	Yersinia pestis Sequencing Group
Glucose 6-phosphate dehydrogenase	0	+	Unknown	Loss of hexose monophosphate pathway dictating reversal of transketolase and transaldolase function or reliance on host pentose for synthesis of RNA	Mortlock and Brubaker, 1962

Table 1. Continued

Determinant	<i>Y. pestis</i>	<i>Y. pseudotuberculosis</i>	Mutational event	Consequence in <i>Y. pestis</i> <sup>a</sup>	References
Aspartase	0	+	IS element insert	Stoichiometric conversion of exogenous L-glutamate to L-aspartate with loss of the oxalacetate pool and enhanced requirement for CO <sub>2</sub>	Dreyfus and Brubaker, 1978
Biosynthesis of glycine (or L-threonine)	0 <sup>b</sup>	+	Possible loss of serine hydroxymethyltransferase by unknown mutational event	Elimination of unnecessary function	Brubaker and Sulen, 1971 Burrows and Gillett, 1966
Biosynthesis of L-valine and L-isoleucine	0	+	Possible loss of regulation by unknown mutational event	Elimination of unnecessary function	Burrows and Gillett, 1966
Biosynthesis of L-phenylalanine	0 <sup>b</sup>	+	Possible loss of regulation by unknown mutational event	Elimination of unnecessary function	Burrows and Gillett, 1966
Biosynthesis of L-methionine	0 <sup>b</sup>	+	Possible loss of homocysteine methyltransferase by unknown mutational event	Elimination of unnecessary function	Burrows and Gillett, 1966 Englesberg and Ingraham, 1957
Fermentation of rhamnose	0 <sup>b</sup>	+	Loss of hydrolase by unknown mutational event	Elimination of unnecessary function	Englesberg, 1957
Fermentation of melibiose	0 <sup>b</sup>	+	Loss of hydrolase by unknown mutational event	Elimination of unnecessary function	Pollitzer, 1954
Urease	0 <sup>b</sup>	+	Frameshift	Elimination of unnecessary function	Sebbane et al., 1999
Assimilation of low levels of NH <sub>4</sub> <sup>+</sup> into organic linkage	0 <sup>b</sup>	+	Possible loss of glutamine synthase by unknown mutational event	Elimination of unnecessary function	Brubaker and Sulen, 1971

Abbreviations: pPCP, pesticin-producing plasmid; +, present; 0, absent; Yops, yersinia outer proteins; pMT, metallothionein plasmid; Pgm, pigmentation phenotype; *hms*, hemin storage locus; *YadA*, yersinia adhesin phenotype; *Inv*, a host cell invasin; *Ail*, another host cell invasin.

<sup>a</sup>These issues are considered further in sections concerned with Phylogeny, Physiology, and Disease.

<sup>b</sup>Known to undergo meiotrophic reversion to the *Y. pseudotuberculosis* phenotype.

further work revealed the presence of additional IS285 (Filippov et al., 1995) and IS200-like (Simonet et al., 1996) inserts. At least 15 copies of the latter, now termed “IS154I,” exist in the chromosome (Odaert et al., 1998; Simonet et al., 1996). Recognition of these elements in *Y. pestis* initially prompted the specter of chromosomal instability due to the occurrence of continuous insertion and excision. However, many of the inserts lack inverted terminal repeats, and it is now believed that some, if not all, are immobile (Odaert et al., 1998). Nevertheless, their widespread distribution within the genome has important consequences. One such outcome is that their existence, especially in close proximity, can result in reciprocal recombination accompanied by loss of intervening DNA. This situation was observed first by Fetherston et al. (1992) for the high-frequency deletion of the pigmentation (*pgm*) locus (Brubaker, 1970a). As defined under Physiology, this 102-kb deletable sequence contains a ~35-kb high-pathogenicity island encoding functions required for synthesis of the siderophore yersiniabactin and a linked ~6-kb hemin storage (*hms*) locus necessary for blocking the flea proventriculus. In nature, deletion of this region obviously is lethal to *Y. pestis*. However, analogous but nonlethal recombinational events between IS elements conceivably may have occurred during the evolution of the species, resulting in the formation of large chromosomal inversions or transpositions without net loss of DNA. Indeed, reciprocal recombination between IS elements may account for the observed integration and excision of plasmids. A comparison of the order of genes in fully sequenced chromosomes of *Y. pestis* and *Y. pseudotuberculosis* will be instrumental in determining the role, if any, played by internal chromosomal rearrangement in the emergence of plague.

Of possible additional importance in the expression of acute disease is the generation of mutations as a direct consequence of IS insertion within the chromosome. The total genome of *Y. pestis* is 4,389.5 kb as judged by two-dimensional pulsed-field gel electrophoresis (Lucier and Brubaker, 1992). Assuming an approximate length of 700 bp for each of the ~50 inserts present in the species, it is evident that almost 0.1% of the genome is composed of these elements. The sequence of sites preferred for insertion is not fully resolved but convincing evidence has accrued indicating that IS154I may prefer stem-loop structures resembling  $\theta$ -independent transcription terminators (Odaert et al., 1998). Genes in *Y. pestis* known or suspected to have undergone inactivation by IS elements resulting in identified changes of phenotype are noted in Table 1. Two of these encode the host cell inva-

sins *Inv* and *Ail*, which are established important virulence factors of the enteropathogenic yersiniae (Finlay and Falkow, 1997). Aspartase also may have undergone inactivation by insertion. Disruption by IS elements is not, of course, the only mechanism that caused loss of structural genes during the emergence of *Y. pestis*. An alternative process is the occurrence of errors in DNA replication evidenced by addition or deletion of bases or sequences within runs of the same base or sequence. Examples of these frameshift mutations are loss of the ability to express chromosomally encoded urease (Sebbane et al., 1999) and *YadA*, which are encoded is a pseudogene on pCD (Rosqvist et al., 1988). Like *Inv* and *Ail*, *YadA* is an important virulence factor required by enteropathogenic yersiniae for gastrointestinal invasion (Finlay and Falkow, 1997; Ruckdeschel et al., 1996). A similar but phenotypically undefined event is the mutation to *YlpA*<sup>-?</sup>, also encoded as a pseudogene on pCD (Hu et al., 1998; Perry et al., 1998).

It is not yet certain whether the mutations listed in Table 1 occurred by accident or design. That is, was a determinant so dispensable that its random loss in an early clone did not reduce fitness or was its removal from the genome to provide a selective advantage? Probably both mechanisms were at work during the emergence of *Y. pestis*. For example, it is difficult to imagine how the ability to ferment an uncommon sugar (such as rhamnose or melibiose) would assist an organism restricted to an environment (the nutritionally rich mammalian host or flea vector) probably lacking significant levels of these carbohydrates. Nevertheless, these traits might benefit an enteropathogenic species, when between hosts, to compete with saprophytes in austere natural environments for lengthy periods. The same argument holds for the ability to assimilate low levels of NH<sub>4</sub><sup>+</sup>, hydrolyze urea or synthesize amino acids. All of these properties favor maintenance in soil or water but are redundant in host fluids or the gorged flea vector. Indeed, pioneer studies undertaken with *Y. pestis* designed to correlate auxotrophy and avirulence demonstrated that the ability to synthesize many amino acids, pyrimidines and vitamins is dispensable, and only nutritional dependence on exogenous purines prevents sustained growth in vivo (Burrows, 1960; Burrows, 1963; Burrows and Gillett, 1966). On the other hand, loss of other traits shared by *Y. pseudotuberculosis* and *Y. enterocolitica* might facilitate the rapid invasion of tissues characteristic of plague bacilli. For example, the determinants *YadA*, *Inv* and *Ail* promote penetration of the intestinal tract by the enteropathogenic yersiniae and, as such, serve as host cell adhesins (Finlay and Falkow, 1997). These activities, however, would become unnec-

essary, if not a liability, upon acquisition of the pPCP-encoded ability of Pla to initiate an intradermal or subcutaneous infection following administration by flea bite. That is, selective pressure favoring the ability of Pla to promote rapid dissemination to visceral organs, known to serve as favored niches for vegetative growth (Nakajima et al., 1995; Straley and Cibull, 1989; Une and Brubaker, 1984a; Une and Brubaker, 1984b; Une et al., 1986), would reward mutational loss of YadA, Inv and Ail. In this context, it is significant that loss of YadA in *Y. pseudotuberculosis* was reported to increase virulence (Rosqvist et al., 1988) as might be expected if YadA inhibited bacterial dissemination in tissues. Unfortunately, these results could not be repeated (Han and Miller, 1997), but further study may show that introduction of YadA into *Y. pestis* can reduce infectivity.

The advantage of removing adhesins that would otherwise serve as anchors impeding tissue invasion is readily understood. In contrast, the prospect that elimination of enzymes central to intermediary metabolism (such as glucose 6-phosphate dehydrogenase or aspartase) also might favor the occurrence of acute disease is more subtle. These enzymes are central to efficient intermediary metabolism in complex enriched media. For example, loss of glucose 6-phosphate dehydrogenase blocks the hexose monophosphate pathway, thereby favoring the assimilation of gluconate or ribose in vivo (Mortlock, 1962a; Mortlock and Brubaker, 1962b). Interconvertible tricarboxylic acid cycle intermediates are lost as excreted aspartate in aspartase-deficient *Y. pestis* (Dreyfus and Brubaker, 1978), which accounts, in part, for the increased doubling time and reduced terminal population of *Y. pestis* grown in vitro as compared with *Y. pseudotuberculosis*. However, the fact that these enzymatic deficiencies are deleterious to *Y. pestis* in vitro by no means indicates that their mutational losses similarly reduce fitness in vivo. The consequences of the absence of glucose 6-phosphate dehydrogenase and aspartase in *Y. pestis* are considered in greater detail under Physiology and Disease.

It is of considerable historical interest that many, but not all, of the traits expressed by *Y. pseudotuberculosis*, but not *Y. pestis*, can be recovered by use of appropriate selective media. This phenomenon was first demonstrated by Englesberg (1957a), who isolated rare mutants (termed "meiotrophs") capable of fermenting rhamnose from otherwise typical cells of *Y. pestis*. Results of similar studies showed that other traits generally absent in *Y. pestis* including urease, and the ability to assimilate low levels of  $\text{NH}_4^+$ , synthesize certain amino acids, and ferment melibiose (Table 1) also could be restored

by meiotrophic reversion (Brubaker and Sulen, 1971; Englesberg and Ingraham, 1957b). It may be significant that all of these cases involve loss of a chromosomally encoded gene not essential for growth in either the flea vector or mammalian host. As already noted, loss of urease involves a single base deletion (Sebbane et al., 1999) typical of rare mutations in DNA replication. This type of forward mutation can readily undergo true reversion; inspection of the sequenced genome may reveal that the loss of other nonessential traits subject to meiotrophic reversion reflects similar occurrence of uncommon errors in replication.

Isolates of many but not all serotypes of *Y. pseudotuberculosis* also possess the high-pathogenicity island encoding yersiniabactin, although their location in the chromosome differs from that in *Y. pestis* (Buchrieser et al., 1998a; Buchrieser et al., 1999; Hare et al., 1999b). Likewise, DNA of *Y. pseudotuberculosis* contains IS elements identical to those of *Y. pestis*, although the number of total copies is considerably smaller (Devalckenaere et al., 1999; Hare et al., 1999b; Odaert et al., 1996). Based on this information and that provided previously, the following scenario may define the emergence of *Y. pestis*. First, a clone of *Y. pseudotuberculosis* capable of yersiniabactin synthesis and hemin absorption procured CafI and murine toxin presently encoded by pMT. It is understood that this addition need not necessitate direct transformation of pMT per se but could involve acquisition of the two determinants by other, perhaps even independent, steps. Supporting this notion is the observation that the operon mediating expression of CafI is an evident high-pathogenicity island replete with flanking bacteriophage homologs (Hu et al., 1998; Lindler et al., 1998). Acquisition of CafI and especially murine toxin, which are known to facilitate survival of yersiniae within the flea midgut (Hinnebusch et al., 1998a; Hinnebusch et al., 1999), would irreversibly change the hereditary destiny of *Y. pseudotuberculosis* by providing a new selective pressure leading inevitably to the emergence of lethal disease. These influences are threefold. First, the recipient early clone would become protected within a closed flea-host-flea cycle and thus be released from the necessity of competing with saprophytes in natural environments. Accordingly, mutational loss of traits that favor survival in austere environments could occur with impunity. Second, the transformed bacterium would become dependent upon the flea for transfer to new hosts and therefore subsequent genetic changes that favor death of the old host would provide a selective advantage. Third, expression of the Hms<sup>+</sup> phenotype, known to be potentially detrimental in vitro (Brubaker,

1970b), now could become constitutive at 26°C, thereby, furthering fitness within the flea.

The newly evolved mechanism of bacterial transfer by fleabite rewarded later mutational events favoring acute disease because death assures that resident fleas will disembark in search of new hosts. This strategy, of course, is distinct from that of *Y. pseudotuberculosis* where expression of a chronic enteropathogenic infection favors prolonged and extensive release of bacteria into natural environments. In this context, it is significant that mice infected intravenously with *Y. pestis* succumb to infection earlier and exhibit a lower bacterial burden in visceral organs than do mice similarly infected with *Y. pseudotuberculosis* (Nakajima et al., 1995; Une and Brubaker, 1984a). The acquisition of pPCP-encoded Pla, known to facilitate tissue invasion (Brubaker et al., 1965; Lahteenmaki et al., 1998; Sodeinde et al., 1992), probably occurred after establishment of the flea-host-flea cycle as did mutational loss of *YadA*, *Inv* and *Ail*, which were previously required by the *Y. pseudotuberculosis* parent for penetration and colonization of the host gastrointestinal tract. These determinants were no longer essential for invading the viscera, which now was readily accessed following infection by fleabite via the action of Pla. Indeed, the three activities possibly restricted the Pla-dependent process now utilized by the emerging plague bacillus for dissemination from peripheral sites of infection to the viscera. Furthermore, *YadA*, *Inv* and *Ail* all promote intimate association with both professional and nonprofessional phagocytes, a process that would be expected to upregulate proinflammatory cytokines, thereby alerting the host to the advent of systemic disease. As noted under Disease, the hallmark of plague is downregulation of IFN- $\gamma$  and TNF- $\alpha$  (Nakajima and Brubaker, 1993); thus, loss of factors capable of upregulating expression of these and other proinflammatory cytokines would become favored. Further work will be required to determine if elimination of glucose 6-phosphate dehydrogenase and of aspartase also contributes to expression of acute disease. As noted under Physiology, removal of these activities might very well alter the local amino acid pools of the host, thereby actually increasing the efficiency of catabolism *in vivo*.

Evident early endemic forms of *Y. pestis* remain in the ancient plague reservoirs of Russia. These "Pestoides" isolates differ from the epidemic forms of the species in that pPCP is not required for tissue invasion (Samoilova et al., 1996; Welkos et al., 1997). A definitive comparison of the genome of these isolates to those of *Y. pseudotuberculosis* and typical epidemic forms of *Y. pestis* may help in unraveling the evolutionary pathway to plague. In summary, the

epidemic form of *Y. pestis* now resident in sylvatic foci throughout the world has evolved as a rare exception to the epidemiological tenet of Theobald Smith (Smith, 1934). This principle states in essence that it is ruinous strategy for a parasite to seriously debilitate its host because, by so doing, that parasite threatens the only environment capable of supporting its own existence in nature. Smith's premise clearly applies to the enteropathogenic yersiniae where maintenance of a chronic low-grade infection favors transmission of the agents to new hosts via fecal contamination. However, acquisition of a more certain route of transfer (i.e., fleabite) mediated by the *hms* locus (Hinnebusch et al., 1996) and pMT (Hinnebusch et al., 1998b; Hinnebusch et al., 1999) favored selection for lethality because death is required to assure the departure of the vector in search of a new host. Once this mechanism of transfer became fixed, additional hereditary changes such as acquisition of pPCP and loss of *YadA*, *Inv* and *Ail* provided increased fitness, which facilitated emergence of the most destructive epidemic disease experienced by humankind. This concept is cavalier in that it ignores the prospect that the high-pathogenicity island encoding yersiniabactin synthesis has evidently undergone transfer to yersiniae on separate occasions (Buchrieser et al., 1998a; Hare et al., 1999b). It further overlooks the finding that the bacteriocin pesticin, also encoded on pPCP (Ferber and Brubaker, 1981), utilizes as a target the yersiniabactin-receptor *Psn* encoded within the high-pathogenicity island (Fetherston et al., 1995; Fetherston and Perry, 1994; Lucier et al., 1996; Rakin et al., 1996; Sikkema and Brubaker, 1987) suggesting prior association between *psn*, linked *pla*, and the chromosomally encoded high-pathogenicity island. The scenario only peripherally addresses the possibility that known changes in intermediary metabolism caused by loss of glucose 6-phosphate dehydrogenase and of aspartase contribute to the severity of disease. Finally, the ability of Pestoides variants to undertake Pla-independent invasion of tissues is overlooked. Again, direct comparison of the fully sequenced genomes of *Y. pestis* and *Y. pseudotuberculosis* should resolve the nature and order of genetic changes accounting for the emergence of plague. Divergence of *Y. pestis* has continued after its emergence; properties used to distinguish biovars of the species are discussed under Epidemiology.

## Taxonomy

*Yersinia* (constituting Genus XI of the Family Enterobacteriaceae) includes *Y. pestis* as the type species, enteropathogenic *Y. pseudotuber-*

*culosis* and *Y. enterocolitica*, *Y. ruckeri* (a primary pathogen of fish), and secondary invaders or saprophytes (*Y. aldovae*, *Y. bercovieri*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. mollaretii* and *Y. rohdei*). Only the first three listed species share pCD/pYV required for expression of primary disease in mammals (Brubaker, 2000; Perry and Fetherston, 1997). Both *Y. pestis* and *Y. pseudotuberculosis* exhibit a 46 to 47 mol% G+C content as opposed to values of 47 to 48.5 established for DNA of *Y. enterocolitica*. Under stringent conditions of reassociation, DNA of *Y. pestis* exhibited 9, 23 and 83% homology to that of *Escherichia coli*, *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively; hybridization with tested species classified outside of the Enterobacteriaceae was not significant (Moore and Brubaker, 1975). The hybridization of DNA under more permissive conditions and biochemical characterization by Dr. H. H. Mollaret and colleagues at the Institut Pasteur, Paris, verified this relationship and provided the basis for defining the additional eight species of the genus (Bercovier et al., 1980; Brenner et al., 1976). Determinations of restriction fragment length polymorphism by Achtman et al. (1999) indicated that *Y. pestis* diverged from *Y. pseudotuberculosis* only 1,500 to 20,000 years ago but that their progenitor separated from *Y. enterocolitica* at least 41 million years earlier. The similarity in gene order between pCD and pYV of *Y. pseudotuberculosis* as opposed to that of pYV from *Y. enterocolitica* (Hu et al., 1998) further substantiates the close relationship between *Y. pestis* and *Y. pseudotuberculosis*. As judged by analysis of 16S rRNA, the genus provides a coherent cluster where *Y. pestis*, *Y. pseudotuberculosis* and certain isolates of *Y. kristensenii* represent one subline, and *Y. enterocolitica* forms another (Ibrahim et al., 1993).

## Habitat

The two major environments exploited by *Y. pestis* in nature consist of the flea vector and the mammalian host. At least two genetic elements (the chromosomally encoded *hms* locus and pMT) are required for growth of plague bacilli in the flea proventriculus. On the other hand, full virulence in rodents requires carriage of pCD, pPCP, pMT, a chromosomal high-pathogenicity island, and a modest number of additional chromosomal genes shared by *Y. pseudotuberculosis* (Achtman et al., 1999; Brubaker, 1991; Brubaker, 2000; Perry and Fetherston, 1997). Functions required for survival in these niches and their regulation are described under Physiology; the effect of virulence factors on the host is defined in Disease. As noted under Phylogeny,

the ability to thrive in both the flea vector at room temperature and host at 37°C probably reflects lateral transfer of extrachromosomal elements during the recent evolution of the species. Plague bacilli also have been isolated from soil recovered from the depths of burrows of social rodents recently exterminated by the disease (Bahmanyar and Cavanaugh, 1976). Whereas the ability to survive in this circumstance may facilitate infection of new occupants, plague bacilli are poorly equipped for competition with saprophytes and rapidly disappear from most natural environments (Poland and Barnes, 1979).

## Isolation

During the course of epizootics, cells of *Y. pestis* can readily be isolated in essentially pure culture from tissues or fluids obtained from the moribund or recently deceased rodent host. In this situation, tissue from the liver or spleen, as well as blood from the heart, typically contain large numbers of yersiniae and are favored sources for subculture. Material aspirated directly from buboes of human patients also is rich in plague bacilli as is sputum obtained from victims of the pneumonic form of the disease. The organisms also have been isolated from fleas removed from rodents captured in epizootic foci. One technique used to facilitate this process is to stun the fleas present on a captured host by brief exposure to chloroform vapor and then macerate these vectors with a sterile glass rod. The resulting debris can then be enriched by inoculation into mice or even streaked directly onto the surface of any of a number of solid enriched commercial media and incubated at room temperature for 2 days. Blood agar base or heart infusion agar is often used for routine isolation and cultivation of *Y. pestis* and the enteropathogenic yersiniae. All of the above sources can yield confluent growth on solid media at the initial site of inoculation; bacteria isolated in pure culture from single colonies should be subcultured at room temperature to avoid possible population shifts accompanied by avirulence caused by loss of pCD. Detailed information is available for epidemiologists and clinicians concerned with primary isolation of *Y. pestis* in the field (Bahmanyar and Cavanaugh, 1976; Butler, 1983; Poland and Barnes, 1979; Pollitzer, 1954).

## Identification

Identification of *Y. pestis* is undertaken at three levels depending upon urgency and the nature of determinative reagents that are on hand. Level one concerns tentative identification in the field;



level two is verification of the preliminary determination with a specific immunological assay; and level three involves use of modern, highly sensitive procedures involving specific molecular probes. The first case of an outbreak is typically the most difficult but most important one to recognize. Unfortunately, clinicians in rural areas where the disease is not anticipated often lack specific reagents necessary for making a correct diagnosis. In this situation, it is imperative that the possibility of plague be considered if a patient has a fever accompanied by a swollen and tender regional lymph node (bubo) or bloody sputum. In the case of plague, fluid recovered from these sources typically contains numerous bacteria visible in direct smears as small stout Gram-negative rods. This material can be used to isolate the organisms on solid medium, tested directly by fluorescent antibodies, or prepared for analysis with specific antigen or oligonucleotide probes. Epidemiologists and public health workers utilize pooled flea detritus as a favored source of yersiniae; this material can be enriched by injection into mice.

In almost all cases, positive identification of *Y. pestis* is dependent upon recognition of at least one of the unique determinants listed in Table 1. A formal determination of *Y. pestis* or diagnosis of plague is dependent upon demonstrating specific genes or gene products. Nevertheless, clinicians lacking specific reagents can still provide convincing evidence that suggests a suspicious organism is indeed *Y. pestis*. Isolates falling into this category should be forwarded to a regional public health facility in possession of specific reagents for further analysis and verification.

Properties used to characterize yersiniae in the field are discussed under Phylogeny and Physiology; their determinative potential was defined by Surgalla et al. (1970). These factors are the abilities of *Y. pestis* to grow as Pgm<sup>+</sup> colonies at 26°C on hemin (Jackson and Burrows, 1956a) or Congo red agar (Hare and McDonough, 1999a; Surgalla and Beesley, 1969), elaborate Pla as judged by use of fibrin plates or rabbit plasma (Beesley et al., 1967), produce the bacteriocin pesticin capable of killing serotype IA or IB cells of *Y. pseudotuberculosis* on solid medium (Brubaker and Surgalla, 1961), and exhibit a  $-10^4$ -fold reduction in ability to form colonies on magnesium oxalate agar at 37°C as opposed to 26°C (Higuchi and Smith, 1961). The obvious disadvantage of these methods is they depend upon prior isolation of the bacteria in pure culture, although hemin or Congo red agar may be used for this purpose. Indeed, any Gram-negative rod recovered from a patient exhibiting clinical symptoms suggestive of plague (as defined under Disease) should be suspected of being *Y. pestis*, if the isolate requires 2 days to

form visible pigmented colonies. Further demonstration of fibrinolytic activity (or coagulation of rabbit plasma), a temperature-dependent differential in ability to form colonies on magnesium oxalate agar, and inhibition of *Y. pseudotuberculosis* in an agar overlayer strongly suggests that the isolate is *Y. pestis* (Surgalla et al., 1970). Another novel test for *Y. pestis* is constitutive expression of isocitrate lyase, a component of the glyoxylate bypass (Quan et al., 1982). Those Pgm<sup>+</sup> isolates possessing any of the additional properties noted above should be handled with caution and are worthy of submission to a central laboratory capable of specific diagnosis.

Clinical specimens suspected of containing *Y. pestis* can be determined immediately in regional health facilities by use of a standardized fluorescent antibody procedure dependent upon detection of capsular Caf1; this and other serological techniques (including the widely used passive hemagglutination test) are described by Butler (1983). The latter also is dependent on expression of Caf1 and has traditionally been used to verify diagnosis of the organism; this procedure is still viewed by many workers as the gold standard for diagnosis of plague. However, a number of more sensitive molecular determinations based on the use of specific antigen (Butler, 1983) or oligonucleotide probes have recently been developed. Extremely sensitive methods that utilize oligonucleotide targets to unique determinants are now used in many public health centers routinely to screen clinical specimens received from the field. Paramount in this effort was the use of polymerase chain reaction (PCR) for amplification of DNA sequences capable of interacting with probes prepared from *pla*, *caf1* or both (Campbell et al., 1993; Hinnebusch and Schwan, 1993; Norkina et al., 1993; Tsukano et al., 1996). Another such method that does not rely on unique structural genes is the procedure of Trebesius et al. (1998), which depends upon amplification of a 23S rRNA sequence specific to *Y. pestis*. This technology successfully identified sequences of *pla* and *Y. pestis*-specific *rpoB* ( $\beta$ -subunit of RNA polymerase) in ancient DNA recovered from dental pulp of plague victims of the second pandemic in France (Drancourt et al., 1998). Detection in fleas using PCR proved superior to inoculating mice (Engelthaler et al., 1999). Indeed, a Q-PCR procedure designed to detect *pla* provided valuable information regarding the distribution of blocked versus merely infected fleas in the aftermath of a naturally occurring epizootic (Hinnebusch et al., 1998a). Second generation procedures designed to ascertain hybridization of specific probes have increased the sensitivity of detection. For example, use of a fluorescently labeled oligonucleotide probe to determine Pla via “*TaqMan*”

technology permitted specific detection of 210,000 copies of the target (1.6 pg of bacterial DNA) in clinical samples (Higgins et al., 1998). Similarly, use of a branched-chain DNA assay to detect pla in concert with an enzyme-linked immunosorbent assay (ELISA) and light addressable potentiometric sensor formats yielded detection limits of 10,000 and 1,000 targets, respectively (Iqbal et al., 1999).

Although Pla is clearly specific for *Y. pestis*, it is a close analog of OmpT of *E. coli* (Sodeinde and Goguen, 1989), an outer membrane protein shared by many enteric bacteria. Accordingly, sequences must be chosen carefully when designing a probe that can specifically recognize *pla*. The bacteriocin pesticin, also encoded on pPCP (but by *pst*), was not shared by other tested bacteria (Vollmer et al., 1997); thus, it may prove to be a superior specific target. This prospect was supported by the finding that only 3 copies of *pst* targets were sufficient to yield a positive reaction by *TaqMan* analysis (Iqbal et al., 2000). Unlike results obtained with *pla* targets (Higgins et al., 1998), those reported for *pst* targets were not influenced by thermal cycle number, suggesting that pesticin is very specific for *Y. pestis* (Iqbal et al., 2000).

## Cultivation

Plague bacilli can multiply between pH 5.5 to 9.0 at temperatures ranging from about 4 to 42°C; doubling times are markedly decreased by aeration. The effect of temperature on the nutrition and phenotype of *Y. pestis* is remarkable and undoubtedly reflects distinct adaptations favoring survival in the flea vector and host. The bacteria are robust during growth at -26°C and capable of achieving extraordinarily high terminal populations in enriched media (Higuchi and Carlin, 1957). The organisms exhibit a nutritional requirement at 26°C for sulfite, thiosulfate, or L-cysteine as a source of sulfur (Englesberg and Ingraham, 1957b) plus exogenous L-threonine (replaceable by glycine), L-methionine, L-isoleucine, L-valine and L-phenylalanine (Brubaker and Sulen, 1971; Burrows and Gillett, 1966). The need for L-isoleucine, L-valine and L-phenylalanine is not absolute and examination of the sequenced genome may reveal that these lesions reflect loss of regulatory functions. The addition of one of the three sources of sulfur also provides reducing potential that favors colony formation on solid medium; sodium thiosulfate (2.5 mM) is often used for this purpose, although hemin also may be employed (Herbert, 1949). The organisms evidently cannot assimilate low levels of  $\text{NH}_4^+$  into organic linkage (Brubaker and Sulen, 1971), thus the addition of this cation

to minimal medium at concentrations of 10 mM is recommended. Growth also depends upon a source of fermentable carbohydrate. Although readily metabolized, D-glucose is not suitable for this purpose due to its prompt conversion to organic acids, causing marked reduction of pH. This difficulty is avoided by use of D-gluconate (Brubaker and Surgalla, 1964; Mortlock, 1962a), which is tolerated at concentrations of at least 0.04 M and maintains neutrality throughout logarithmic growth (Fowler and Brubaker, 1994). Consequently, a minimal medium for *Y. pestis* might contain physiological salts ( $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{HPO}_4^{2-}$ ),  $\text{NH}_4^+$  as a source of nitrogen, D-gluconate as a source of carbon and energy, thiosulfate as a source of sulfur and reducing agent, and substrate levels of L-threonine, L-isoleucine, L-valine, L-methionine and L-phenylalanine (Brubaker, 1970a; Brubaker and Sulen, 1971). Growth at 26°C or less (especially in enriched media) may result in aggregation and adherence of Pgm<sup>+</sup> yersiniae to the surface of culture vessels eventually causing emergence of an even suspension composed of avirulent Pgm<sup>+</sup> mutants.

Cultivation at 37°C in chemically defined media is far more exacting and requires the presence of additional nutrients. Media constructed to mimic the composition of mammalian cytoplasm are generally satisfactory for this purpose, although absolute nutritional requirements for amino acids other than those needed for growth at 26°C are difficult to establish. Problems in defining requirements for additional nutrients at host temperature largely stem from the inadvertent introduction of unbalanced or toxic conditions caused by removal of single components. In addition, the possibility exists that functions concerned with vegetative growth at 26°C, especially active transport, are downregulated at 37°C. For many purposes, it is possible to use commercial preparations of natural products such as casein digests or meat infusions for routine growth at 37°C. These media contain peptides that are less toxic than free amino acids and typically can initiate logarithmic growth from smaller inocula than do synthetic media. The chemically defined medium of Higuchi et al. (1959) and its derivatives (Price et al., 1991; Zahorchak et al., 1979) have provided useful information about specific nutritional requirements at 37°C relating to virulence. Paramount among these is a need for  $\text{Ca}^{2+}$  (<2.5 mM) at host temperature but not at 26°C (Kupferberg and Higuchi, 1958). The necessity for  $\text{Ca}^{2+}$  at 37°C is enhanced by -20 mM  $\text{Mg}^{2+}$  (Brubaker and Surgalla, 1964; Higuchi et al., 1959; Higuchi and Smith, 1961) but largely can be eliminated by removing  $\text{Na}^+$  from the medium (Fowler and Brubaker, 1994) and, at least at elevated pH, by adding nucleoside

diphosphates and triphosphates (Zahorchak and Brubaker, 1982). The basis of this unusual nutritional requirement is discussed under Physiology and its consequences in mediating pCD-encoded virulence effectors within the mammalian host are considered under Disease. Organisms starved of  $\text{Ca}^{2+}$  at  $37^\circ\text{C}$  in vitro undergo bacteriostasis, upregulation of pCD-encoded effectors of virulence (i.e., cytotoxic Yops and LcrV), and concomitant reduction in adenylate energy charge (Zahorchak et al., 1979). As a consequence, the bacterial population shifts (Fukui et al., 1957) to reflect selection of avirulent  $\text{Ca}^{2+}$ -independent mutants lacking pCD. A comparison of the kinetics of bacteriostasis in chemically defined medium between *Y. pestis* and the enteropathogenic yersiniae is provided in Fig. 1.

It is generally recognized that the doubling times of *Y. pestis* are significantly greater than those observed for typical enteropathogenic yersiniae in media supplemented with  $\text{Ca}^{2+}$ . This difference largely disappears, however, if the concentration of atmospheric  $\text{CO}_2$  is brought to 10% whereupon single cells of *Y. pestis* can form visible colonies at  $37^\circ\text{C}$  on enriched solid medium in 24 hours rather than the usual 2 days. The concentration of metabolic  $\text{CO}_2$  generated in suitably enriched liquid media is sufficient to permit cells of *Y. pestis* to achieve doubling times during the mid-logarithmic growth phase of about 70 min at both  $26^\circ$  and  $37^\circ\text{C}$ . As shown in Fig. 1, this value approximates that observed for enteropathogenic yersiniae cultivated in the

same medium (Carter et al., 1980). Despite the marked differences in physiology between yersiniae grown at  $26^\circ\text{C}$  versus  $37^\circ\text{C}$ , the organisms respond rather slowly to shifts in temperature and changes in availability of nutrients. For example, a lag phase of many hours occurs upon use of bacteria previously cultivated at  $5^\circ\text{C}$  as inocula for liquid media incubated at higher temperatures, especially  $37^\circ\text{C}$ . The basis for this inefficient regulation is not fully resolved but may reflect deficiencies in global control processes or reliance upon regulatory mechanisms involving quorum sensing. To assure steady state kinetics in liquid cultures, it is therefore imperative to utilize late-logarithmic, growth-phase cells by subculturing them two or three times in the same medium before performing experiments. Claims in the old literature that cells of *Y. pestis* grow more rapidly at room temperature than at host temperature are incorrect and probably reflect ignorance of sluggish regulation, the nutritional requirement for  $\text{Ca}^{2+}$ , and the stimulatory effect of  $\text{CO}_2$ .

## Physiology

### Intermediary Metabolism

Results of early studies indicated that plague bacilli possess complete Embden-Meyerhoff (glycolytic), hexose-monophosphate (pentose phosphate), and tricarboxylic acid (Krebs cycle)

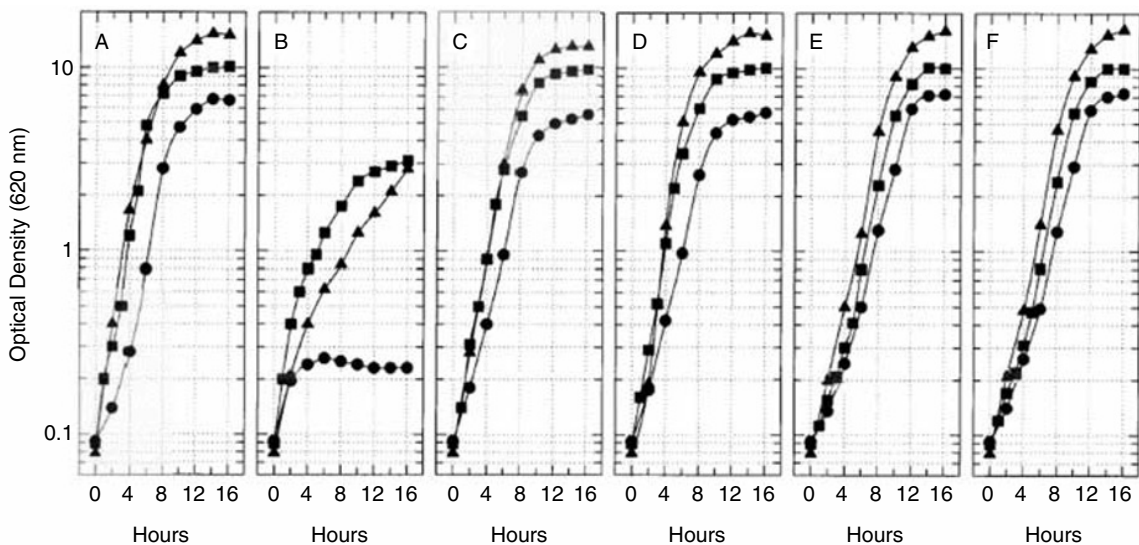


Fig. 1. Growth of *Y. pestis* KIM10 (●), *Y. pseudotuberculosis* PB1 (■), and *Y. enterocolitica* WA (▲) in chemically defined medium (Zahorchak and Brubaker, 1982); incubation of pCD/pYV<sup>+</sup> cells at  $37^\circ\text{C}$  plus (A) 2.5 mM  $\text{Ca}^{2+}$  or without (B) added  $\text{Ca}^{2+}$ , pCD/pYV<sup>-</sup> cells at  $37^\circ\text{C}$  plus (C) 2.5 mM  $\text{Ca}^{2+}$  or without (D) added  $\text{Ca}^{2+}$ , and pCD/pYV<sup>+</sup> cells at  $26^\circ\text{C}$  plus (E) 2.5 mM  $\text{Ca}^{2+}$  or without (F) added  $\text{Ca}^{2+}$ . Significant concentrations of pCD/pYV-encoded virulence effectors and the attendant translocation apparatus are only expressed in vitro during restriction of vegetative growth at  $37^\circ\text{C}$  in the  $\text{Ca}^{2+}$ -deficient medium (B). Data from Carter et al. (1980).

pathways. As noted in later reviews (Brubaker, 1991; Brubaker, 1972; Perry and Fetherston, 1997), all three of these suppositions may be incorrect. For example, attempts to detect glucose 6-phosphate dehydrogenase in *Y. pestis* were not successful under conditions that yielded positive results for *Y. pseudotuberculosis* (Mortlock, 1962a; Mortlock and Brubaker, 1962b). This enzyme is central to the hexose-monophosphate pathway, and its absence necessitates *in vitro* synthesis of pentose from hexose by reversal of the normal physiological reactions catalyzed by transketolase and transaldolase. The deficiency does not, of course, prohibit synthesis of pentose from D-gluconate, which, incidentally, upregulates a functional Entner-Doudoroff pathway (Mortlock, 1962a). Analogous deficiencies of enzymes required for operation of the Embden-Meyerhoff pathway or tricarboxylic acid cycle have not been reported, but ancillary defects may very well modify the traditional roles of these catabolic systems. For example, cells of *Y. pestis* normally utilize the glyoxylate bypass as judged by expression of constitutive isocitrate lyase activity (Hillier and Charnetzky, 1981; Quan et al., 1982). The rationale for this modification of the tricarboxylic acid cycle is unknown. One possibility is that, despite early claims to the contrary, the organisms lack one or more enzymes of this pathway and therefore rely on the glyoxylate bypass to assure cycling of carbon (i.e., regeneration of oxalacetate). In favor of this notion is the observation of the very modest ability of extracts of *Y. pestis* to convert  $\alpha$ -ketoglutarate to succinate (R. Brubaker, unpublished observations). Alternatively, because the glyoxylate bypass typically serves to generate tricarboxylic acid cycle intermediates such as malate from two-carbon fragments (i.e., acetate), it may function in *Y. pestis* to restore oxalacetate (lost upon transformation to L-aspartate). Levels of pyruvate kinase, an enzyme of the Embden-Meyerhoff pathway, also were modest in *Y. pestis* as opposed to *Y. pseudotuberculosis* (R. Brubaker, unpublished observations). Again, as noted below, this result may reflect adjustment of intermediary metabolism to accommodate loss of the oxalacetate pool during growth *in vitro*.

An appreciation of the central role of aspartase in bacterial catabolism is essential in understanding the critical consequences of its mutational loss in *Y. pestis*. This enzyme catalyzes the conversion of L-aspartate to the tricarboxylic cycle intermediate fumarate, which is then converted to malate before undergoing dehydrogenation in a reaction yielding NADH and oxalacetate. The latter fulfills a central role in intermediary metabolism by serving as a substrate in reactions essential to catabolism (formation of citrate for use in the

tricarboxylic acid cycle) and anabolism (formation of the aspartate and pyruvate families of amino acids). It is now recognized that equilibria favoring accumulation of oxalacetate generally stimulate growth of plague bacilli *in vitro*, whereas equilibria facilitating oxaloacetate destruction are detrimental. For example, Baugh et al. (1964) found oxalacetate to be the primary product of CO<sub>2</sub> fixation, thereby providing an explanation for the ability of this gas to stimulate growth. On the other hand, L-glutamate hastened restriction of plague bacilli cultivated in Ca<sup>2+</sup>-deficient medium (Fowler and Brubaker, 1994) because the organisms can transaminate the oxalacetate pool to L-aspartate, which is then excreted into the supernatant fluid (Dreyfus and Brubaker, 1978). L-proline caused similar excretion in cells of *Y. pestis* but accumulation of L-aspartate did not occur in cultures of *Y. pseudotuberculosis* because the amino acid is promptly recycled into the tricarboxylic cycle as fumarate via the action of aspartase (Fig. 2). In view of these observations, it may be significant that reduced pyruvate kinase activity in *Y. pestis* grown with D-gluconate would promote accumulation of pyruvate, a substrate for the pyruvate carboxylase-mediated fixation of CO<sub>2</sub> into oxalacetate. As already noted, constitutive expression of the glyoxylate bypass would similarly favor restoration of the oxalacetate pool from two-carbon fragments.

Loss of metabolic carbon in the form of excreted L-aspartate accounts at least in part for the smaller number of plague bacilli found during stationary phase after growth under permissive conditions than terminal populations typically achieved by the enteropathogenic yersiniae (Fig. 1). Excretion of L-aspartate also contributes to the abrupt restriction of growth observed for cells of *Y. pestis* harboring pCD following shift to 37°C in Ca<sup>2+</sup>-deficient medium. This prompt shutoff differed significantly from that observed for enteropathogenic yersiniae carrying pYV, which continued to multiply but at a slower rate (Fig. 1). However, a pattern of restriction essentially identical to that shown for the enteropathogenic yersiniae was obtained for *Y. pestis* by removing either L-glutamate or Na<sup>+</sup> from the medium (Fowler and Brubaker, 1994). The role of Na<sup>+</sup> in this context is not fully resolved but it is probably significant that this cation functions as an obligatory antiporter for transport of L-glutamate (Fujimura et al., 1983; MacDonald et al., 1977). In summary, loss of metabolic carbon in the form of L-aspartic acid seriously handicaps plague bacilli during cultivation *in vitro*, especially at 37°C in the absence of Ca<sup>2+</sup>. This catabolic lesion resulting from species-specific mutational loss of aspartase need not, of course, cause loss of fitness *in vivo*. That is, host

Fig. 2. Degradation of L-glutamic acid (A) by resting cells of *Y. pseudotuberculosis* PB1 (▲) and *Y. pestis* EV76 (●) with accumulation of L-aspartic acid (■) by the latter; degradation of L-proline (B) by resting cells of *Y. pseudotuberculosis* PB1 (▼) and *Y. pestis* EV76 (●) with accumulation of L-aspartic acid (■) and L-glutamate (▲) by the latter. The reaction mixture contained  $-5$  mg yersinia,  $33$   $\mu$ moles Tris  $\cdot$  HCl (pH 8.5),  $1.0$   $\mu$ mole  $Mg^{2+}$ , and  $10$   $\mu$ moles of radioactive amino acid ( $0.1$   $\mu$ Ci/ $\mu$ mole) in a volume of  $1.0$  ml; data from Dreyfus and Brubaker (1978).

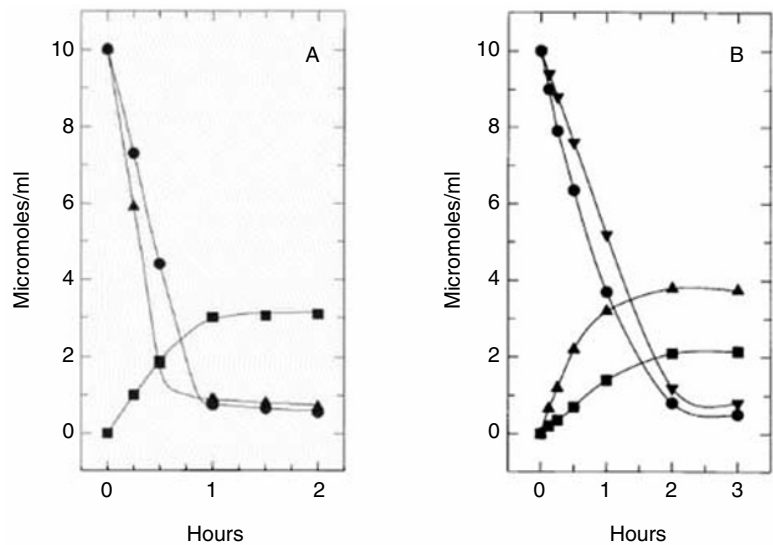


Table 2. Initial rates of destruction of some catabolizable amino acids by resting cells of *Y. pestis* EV76 and *Y. pseudotuberculosis* PB1 in  $0.033$  M MOPS buffer, pH  $7.0^a$ .

Amino acid	Rate of destruction (nmol of amino acid/min/mg dry weight)		Ratio
	<i>Y. pestis</i>	<i>Y. pseudotuberculosis</i>	
L-Aspartate	0	31.0	>31
L-Asparagine	6.4	46.6	7.3
L-Glutamate	2.5	10.2	4.1
L-Glutamine	0	2.2	>2.2
L-Serine	243.8	53.1	0.2
Glycine	18.1	5.8	0.3
L-Alanine	12.8	9.3	0.7
L-Proline	11.9	11.3	0.9
L-Threonine	7.7	10.1	1.3

Abbreviations: MOPS, 3-(N-morpholino) propane sulfonate. <sup>a</sup>Data from Dreyfus and Brubaker (1978).

enzymes would be expected to regenerate L-glutamate from the excreted L-aspartate at the expense of significant metabolic energy. Further study of this phenomenon is required to determine whether it contributes to expression of acute disease.

A comparison of the ability of *Y. pestis* and *Y. pseudotuberculosis* to catabolize the naturally occurring dicarboxylic amino acids, their amines, and some additional catabolizable amino acids is shown in Table 2. The increased rates of destruction determined for L-glutamate, L-glutamine, L-aspartate and L-asparagine in *Y. pseudotuberculosis* undoubtedly reflects the absence of aspartase in *Y. pestis*. However, the remaining catabolizable amino acids were degraded at similar rates with the exception of L-serine, which was destroyed at an exorbitant pace by plague bacilli (Dreyfus and Brubaker, 1978). This phenomenon results as a con-

sequence of constitutive serine deaminase activity (R. Brubaker, unpublished observations). Unregulated destruction of L-serine may have some bearing on the observation that cells of *Y. pestis* require glycine, which can be obtained from L-threonine, probably via threonine aldolase, but not from L-serine, which is known to be the immediate precursor of glycine in *E. coli* (Brubaker and Sulen, 1971). This observation suggests mutational loss of serine hydroxymethyltransferase or an as yet unsuspected defect at the level of folic acid metabolism. The nature of this and other unresolved peculiarities in the intermediary metabolism of *Y. pestis* will shortly become resolved upon publication of the completed sequence of its genome.

## Global Regulation

Existence in plague bacilli of a functional ferric uptake regulator (Fur) system and cAMP-binding protein sequence for regulation of iron assimilation has been reported (Staggs and Perry, 1992). Mechanisms of macromolecular synthesis in *Y. pestis* have received intensive study. As might be anticipated, no significant modifications or innovations have been reported to exist in these typically conserved processes. Similarly, regulation of macromolecular synthesis evidently remains intact, in that plague bacilli are stringent (Charnetzky and Brubaker, 1982) and capable of ordered shutdown of protein, RNA and DNA synthesis during the onset of bacteriostasis prompted by  $Ca^{2+}$ -starvation at  $37^\circ C$  or by normal entrance into stationary phase (Zahorchak et al., 1979). Nevertheless, global regulation at the level of small molecule metabolism may be defective as judged by the peculiarities in catabolism noted immediately above. In

this context, loss of leucine regulatory protein (Calvo and Matthews, 1994; Newman and Lin, 1995) would account for the uncontrolled destruction of L-serine and for many of the nutritional characteristics and sluggish regulation observed for *Y. pestis*. Inspection of the completely sequenced genome should provide an immediate resolution to these issues. Of special importance in this context was the discovery of genes mediating quorum sensing, including *lux*, in the partially sequenced genome (*Yersinia pestis* Sequencing Group, on-line).

The ability of plague bacilli to provide appropriate responses to temperature has received widespread attention. Major cold-shock proteins (MCSPs) mediate psychrotolerance in many diverse groups of bacteria including yersiniae. The three yersiniae pathogenic to humans (plus *Y. ruckeri*) possess two such cold-inducible chromosomally encoded tandem gene duplications that are able to transcribe both bicistronic (*cspA1/A2*) and monocistronic (*cspA1*) mRNA (Neuhaus et al., 1999). Transcription at 30°C was negligible, but temperature shift to 10°C or lower caused a prompt (at least 300-fold) increase in the bicistronic message and a more modest increase of the monocistronic form. Expression of cold-shock proteins (CspA1 and CspA2) was evident after 10 min, illustrating remarkable promptness of the system that upregulates them.

Less is known about the processes that enable yersiniae to adapt to host temperature. At least two mechanisms account for upregulation of virulence effectors following shift from 26 to 37°C. The first is regulated by LcrF (VirF in *Y. enterocolitica*), a pCD-encoded activator required for transcription of a plethora of structural genes located on the same plasmid (Cornelis et al., 1989; Cornelis, 1993; Lambert de Rouvroit et al., 1992). Temperature-dependent melting of DNA within specific internal domains of lcrF assures its transcription; these domains thus serve as the thermostat (Rohde et al., 1999). Once produced, this AraC-like transcriptional activator (Cornelis et al., 1989) binds to specific sequences upstream of other genes encoded by pCD, thereby assuring their expression (Wattiau and Cornelis, 1994). This process regulates the pCD-encoded type III secretion mechanism and attendant effectors of virulence. However, it does not regulate antigens 3, 4 and 5 (Crumpton and Davies, 1956) because these proteins retain normal thermoregulation following mutational loss of pCD. Antigens 3, 4 and 5 correspond to pMT-encoded Caf1, chromosome-encoded pH 6 antigen, and chromosome-encoded KatY, respectively; their structure and function is described below. Garcia et al. (1999a) found three DNA sequences upstream from *katY* that possessed significant homology with the consensus sequence for LcrF

(Wattiau and Cornelis, 1994). This finding is consistent with the prospect that there is an additional chromosomally encoded LcrF-like transcriptional activator. Furthermore, following shift from 26 to 37°C, thermoregulated mRNA of LcrF-activated functions encoded by pCD took significantly longer to appear than did thermoregulated mRNA of chromosomal genes such as *katY* (Garcia et al., 1999b). Preliminary evidence suggests that the pCD-mediated onset of bacteriostasis at 37°C in Ca<sup>2+</sup>-deficient medium can be hastened by undertaking the temperature shift in spent medium (R. Brubaker, unpublished observations). This phenomenon is consistent with quorum sensing and may provide an explanation for the existence of *lux* in the partially sequenced genome (*Yersinia pestis* Sequencing Group, on-line). To appreciate the role played by Ca<sup>2+</sup> in downregulating LcrF-activated functions while facilitating growth at 37°C, the physiology of protein translocation mediated by the pCD-encoded type III secretion system (described below) must be understood.

### Unique Functions Unrelated to Virulence

The majority of unusual or unique functions produced by plague bacilli are virulence factors that neutralize nonspecific mechanisms of host defense. Nevertheless, the organisms possess a limited number of singular mechanisms that facilitate survival in the flea (the abilities to absorb hemin or Congo red and elaborate murine toxin) and assure retention of pPCP (expression of the bacteriocin pesticin). The physiology of these attributes is considered below.

The *hms* LOCUS. Wild-type cells of *Y. pestis* possess the ability to absorb certain exogenous flat planar molecules including hemin (Jackson and Burrows, 1956b) and Congo red (Surgalla and Beesley, 1969) at temperatures of 26°C or less, which form pigmented or Pgm<sup>+</sup> colonies on solid medium. Comparison of iron reservoirs in Pgm<sup>+</sup> versus Pgm<sup>γ</sup> yersiniae revealed that only the former accumulated significant levels of hemin at the outer membrane and that this deposition failed to occur at 37°C (Perry et al., 1993). This capability is mediated by genes comprising the hemin storage or *hms* locus (Perry et al., 1990) located adjacent to the high-pathogenicity island encoding the ability to synthesize yersiniabactin (Bearden et al., 1997; Buchrieser et al., 1998b; Gehring et al., 1998; Heesemann, 1987). Use of the designation Hms<sup>+</sup> indicates the ability to form pigmented colonies known to require an intact *hms* locus and functional *hmsT* gene defined below. These Hms<sup>+</sup> isolates need not, of course, possess the linked high-pathogenicity island encoding yersiniabactin synthesis (or vice

versa). The *hms* locus comprises an *hmsHFRS* operon (Perry and Fetherston, 1997), although absorption of pigments also requires *hmsT* located outside of the deletable region (Jones et al., 1999). Expression of the Hms<sup>+</sup> trait depends upon formation of a surface complex requiring expression of multiple gene products. The HmsH and HmsR proteins, evident on outer membranes, may mediate the stacking of planar molecules, which accounts for the color and brittle morphology of colonies grown at 26°C with exogenous hemin or Congo red. This phenotype is regulated by Fur despite the absence of salient binding sites near *hmsHFRS*; an appropriate potential Fur-binding site was later mapped near *hmsT* (Jones et al., 1999).

As noted under Disease, the high-pathogenicity island within the deletable *pgm* locus is essential for expression of plague; whereas, the *hms* locus is not (Lillard et al., 1999). However, Kutnyrev et al., (1992) discovered that Pgm<sup>+</sup> mutants were unable to effectively colonize the flea vector and this defect was shown by Hinnebusch et al. (1996) to reflect loss of the *hms* locus, rather than the high-pathogenicity island. The precise roles of HmsH and HmsR in facilitating blockade of the flea proventriculus are not resolved, but the phenomenon clearly involves physical adherence of the bacteria to the surface of the flea gut and to each other. This penchant of Pgm<sup>+</sup> yersiniae to specifically undergo autoagglutination during growth at room temperature is observed in the absence of exogenous pigments and may account for the phenomenon of “stalactite” growth described in the early literature. Small planar molecules other than hemin and Congo red are readily absorbed as evidenced by the accumulation of radioactive guanine on the surface of Pgm<sup>+</sup> but not Pgm<sup>-</sup> organisms. Indeed, the affinity of this purine for the bacterial surface was sufficiently strong to prevent its assimilation into cytoplasm and subsequent conversion to adenine (Brubaker, 1970b). The findings that Pgm<sup>+</sup> yersiniae remain associated during growth as particulate masses and are unable to transport certain planar molecules suggest that expression of the Hms<sup>+</sup> trait outside of the flea may be deleterious. If correct, this notion would explain the finding that almost all strains of *Y. pseudotuberculosis* are Hms<sup>-</sup>, although many possess an intact *hmsHFRS* operon (Buchrieser et al., 1998a; Hare et al., 1999b). The reason why the Hms<sup>+</sup> trait is cryptic in *Y. pseudotuberculosis* is not fully resolved; an obvious possibility is downregulation at the level of *hmsT* (Hare et al., 1999b; Jones et al., 1999).

### Murine Toxin

The *hms* locus serves to “block” the flea proventriculus with aggregated bacteria, which causes

regurgitation and repeated attempts at feeding. Though this phenomenon clearly enhances the possibility of infection, it is also eventually lethal to the vector (Perry and Fetherston, 1997). Murine toxin encoded on pMT is another determinant required for survival within the otherwise hostile environment of the flea midgut (Protsenko et al., 1983; Protsenko et al., 1991). Work by J. Hinnebusch and colleagues (Hinnebusch et al., 1998b; Hinnebusch et al., 1999) indicates that this toxic protein is required for colonization (as opposed to blockage) of the flea midgut. Many workers have assumed that the physiological role of murine toxin is to assure prompt death of murine hosts, thereby guaranteeing departure of the infected flea vector. This capability is considered below in more detail. The discovery that murine toxin is also necessary for survival in the flea was therefore unanticipated by some and provides another example illustrating dual functionality in virulence effectors of *Y. pestis*.

### Pesticin

Ben-Gurion and Hertman (1958) discovered that wild-type strains of *Y. pestis* express a unique bacteriocin active against *Y. pseudotuberculosis* that they termed “pesticin.” Further study showed this inhibition was limited to serotype IA and IB cells of *Y. pseudotuberculosis*, highly invasive serovar O:8 isolates of *Y. enterocolitica*, and certain clinical strains of *E. coli* (including the universal colicin indicator strain  $\theta$  (Brubaker and Surgalla, 1961). It is established that group B colicins utilize siderophore receptors to obtain entrance to sensitive bacteria and their antibacterial activity can be inhibited by exogenous Fe<sup>3+</sup> that downregulates these receptors (Davis and Reeves, 1975). Pesticin was the first bacteriocin shown to exhibit this effect (Brubaker and Surgalla, 1961; Brubaker and Surgalla, 1962) and pesticin-insensitive mutants of *E. coli*  $\theta$  displayed interesting patterns of tolerance but not resistance to group B colicins (Ferber et al., 1979b). However, attempts to characterize a siderophore capable of sharing the pesticin receptor (Psn) were unsuccessful until Heesemann (1987) discovered the elusive yersiniabactin (discussed under Phylogeny and Disease). Studies of the mode of action of pesticin revealed that the bacteriocin generated giant osmotically stable spheroplasts from sensitive bacteria (Hall and Brubaker, 1978). This capability, shown in Fig. 3, is unique among bacteriocins and can be utilized as a determinative assay for *Y. pestis*. Pesticin was purified to homogeneity and shown to exist as a monomeric peptide in two conformer states (Hu and Brubaker, 1974; Hu et al., 1972). The bacteriocin is not an *N*-acetylglucosaminidase as initially reported (Ferber and Brubaker, 1979a);

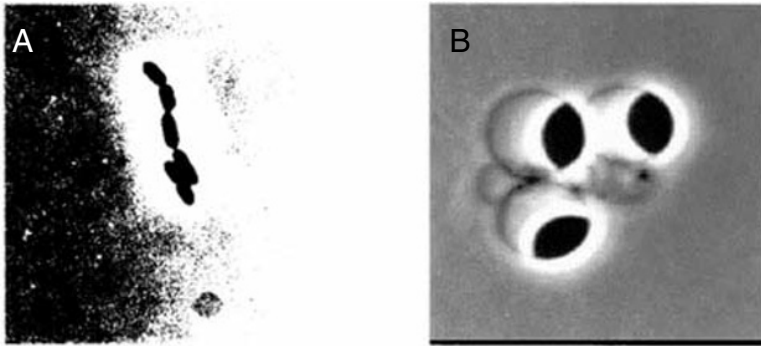


Fig. 3. Morphology of cells of *Y. enterocolitica* after cultivation for 6 h in the absence (A) and presence (B) of homogenous pesticin (40 U per ml); data from Hall and Brubaker (1978).

use of improved methods showed that muramidase activity accounts for peptidoglycan cleavage (Vollmer et al., 1997). Pesticin was initially sequenced by Motin and Brubaker (#U31974) and found to exhibit a molecular mass of 39.9 kDa; a BLAST search failed to yield close homologs. This lack of similarity is in accord with the finding of Vollmer et al. (1997) that the bacteriocin is entirely unique. *Yersinia* capable of producing pesticin are protected by a pPCP-encoded periplasmic immunity protein (PilsI et al., 1996).

**EFFECTORS OF VIRULENCE** As noted under Disease, the virulence factors of *Y. pestis* are concerned with either facilitating bulk vegetative growth in favored visceral niches such as liver and spleen or dissemination of the bacteria to these organs from peripheral sites of infection initiated by fleabite. The chromosome and all three plasmids of the species encode virulence effectors. Their structure and function is described below.

### pCD-Encoded Functions

Most shared virulence factors encoded by pCD/pYV promote cytotoxicity or prevent host reprisal by inhibiting inflammatory reactions required for activation of professional phagocytes. These functions provide the basis for the common symptoms of plague and the localized chronic afflictions caused by the enteropathogenic *Yersinia*. Paramount among these common determinants of virulence is the *Yersinia* outer proteins (Yops), which undergo direct introduction from the cytoplasm of the invading organism into the cytosol of host cells via the pCD/pYV-encoded type III secretion machine. This process of translocation is by injection and depends on docking of the bacterium to the target host cell, thereby minimizing intervention by the immune system. Those Yops that serve as effectors of virulence are often cytotoxic, as judged by their homology with known exotoxins of other species or with established modes of

catalysis. They lack the typical leader sequences of generic outer-membrane proteins, and specific chaperones are required to mobilize their type III secretion (Cornelis, 1998a; Cornelis, 1994; Cornelis et al., 1998b; Cornelis and Wolf-Watz, 1997). However, convincing evidence also has accumulated indicating that at least YopE is recognized and then translocated on the basis of unique non-coding mRNA configuration, rather than primary amino acid structure (Cheng et al., 1997; Lee and Schneewind, 1999). The major Yops were discovered independently in *Y. pseudotuberculosis* by Straley and Brubaker (1981) and in *Y. enterocolitica* by Portnoy et al. (1981b). In wild-type isolates of *Y. pestis*, net synthesis of Yops is not detected in vitro (Chalvignac et al., 1988; Kuttyrev et al., 1999; Mehig and Brubaker, 1993; Mehig et al., 1989; Sample and Brubaker, 1987a; Sample et al., 1987b; Straley and Brubaker, 1981). This difference provides a major distinction between plague bacilli and the enteropathogenic *Yersinia* and is discussed below and under Disease. At least six Yops directly or indirectly promote tissue damage.

The first of these virulence determinants, YopE, causes rounding of nonprofessional phagocytes (e.g., HeLa cells) by disrupting actin microfilaments, exhibits antiphagocytic activity, and is essential for full virulence (Rosqvist et al., 1990; Rosqvist et al., 1991; Straley and Bowmer, 1986; Straley and Cibull, 1989; Straley et al., 1993). In a seemingly unrelated phenomenon, YopE selectively facilitates invasion-dependent cytolysis of lymphoblasts by *Y. pseudotuberculosis* in an RT1-A-restricted, CD8<sup>+</sup> T cell-mediated reaction (Falgarone et al., 1999). A second cytotoxin (YopT) can similarly disrupt the host cell cytoskeleton; unlike YopE, YopT is not essential for lethality (Iriarte and Cornelis, 1998a). An essential virulence factor (YopH; Bölin and Wolf-Watz, 1988; Straley and Bowmer, 1986) has protein-tyrosine phosphatase activity (Bliska et al., 1991; Guan and Dixon, 1990). This effector is also cytotoxic and was reported to prevent assembly of macrophage focal adhesions



required for phagocytosis (Persson et al., 1997) and to block early  $\text{Ca}^{2+}$ -signalling in neutrophils (Andersson et al., 1999). The YopJ determinant (YopP in *Y. enterocolitica*) is essential for full virulence of *Y. pseudotuberculosis* in mice, where it binds directly to salient members of the mitogen-activated protein kinase superfamily (Orth et al., 1999), thereby downregulating TNF- $\alpha$  with concomitant apoptosis of macrophages (Monack et al., 1998; Monack et al., 1997; Palmer et al., 1998; Palmer et al., 1999). This capability was suggested (Mills et al., 1997; Monack et al., 1998; Palmer et al., 1998; Schesser et al., 1998) to account, at least in part, for the remarkable ability of yersiniae to downregulate expression of the major proinflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  (Nakajima and Brubaker, 1993). Nevertheless, YopJ is not essential for lethality of *Y. pestis*, at least in the murine system (Straley and Bowmer, 1986). This discrepancy probably reflects the fact that plague is lethal in mice before significant intervention by macrophages can occur. Further study likely will demonstrate that YopJ also is essential for lethality in more resistant rodents indigenous to natural sylvatic reservoirs, as well as in humans. Mutants lacking YopM also retain significant virulence in mice; the physiological role of this virulence effector and the consequences of its loss in other hosts are unknown (Boland et al., 1996; Nemeth and Straley, 1997). The YpkA determinant (or YopO in *Y. enterocolitica*) is necessary for lethality in the murine model; this protein possesses serine/threonine kinase activity (Galyov et al., 1993). The YopB and YopD determinants are established components of the type III secretion machine, but they also possess independent abilities to form pores in membranes and may thus contribute to host cell necrosis. This effect is dramatic in the case of YopB, which has hemolytic activity (Håkansson et al., 1996).

Since its discovery by T. W. Burrows in 1956 (Burrows, 1956), LcrV (V antigen) has remained in the forefront of yersiniae research. This 37.3-kDa (327-amino acid residue) pure protein (Motin et al., 1992) is unstable and undergoes marked degradation during purification by traditional chromatographic procedures (Brubaker et al., 1987; Motin et al., 1994). Nevertheless, LcrV expressed in *E. coli* in the form of fusion proteins engineered to contain the ligands staphylococcal Protein A (Motin et al., 1994; Nakajima et al., 1995), glutathione S-transferase (Leary et al., 1995), or sequences encoding polyhistidine (Anderson et al., 1996; Motin et al., 1996; Schmidt et al., 1999) can readily be purified to homogeneity by ligand affinity chromatography. Nevertheless, considerable care must still be taken during affinity chromatography to avoid denaturation causing loss of anti-inflammatory

activity and inability to interact with other pCD-encoded proteins; ability to raise protective antibodies is less dependent on maintenance of secondary structure (Motin et al., 1996; Nedialkov et al., 1997). For example, Motin et al. (1996) employed buffers of sufficient ionic strength to avoid nonspecific binding between polyhistidine-linked LcrV and LcrH; failure to avoid this pitfall may have prompted the report that this interaction was an "artifact" (Fields et al., 1997). The ability of LcrV fusion proteins to immunize against plague and to downregulate proinflammatory cytokines is discussed under Disease and Applications. Two additional roles of LcrV are considered below, which involve regulating construction of the pCD-encoded type III secretion machine and targeting the translocation of cytotoxic Yops from docked yersiniae into host cell cytoplasm.

### pMT-Encoded Functions

Structural genes encoding capsular Caf1 antigen (*caf1*) and murine toxin (*ymt*) are encoded by pMT (Protsenko et al., 1983; Protsenko et al., 1991). The former resides within an evident high-pathogenicity island as judged by sequencing the entire plasmid (Hu et al., 1998; Lindler et al., 1998). The primary structure of Caf1 deduced by this process was identical to that first determined upon cloning (Cherepanov et al., 1991; Galyov et al., 1990). Although initially described as a glycoprotein, Caf1 is now recognized as a 15.5-kDa pure protein capable of undergoing polymerization to form a bilayer presenting a hydrophilic exterior surface. These polymers can achieve sizes of many megadaltons (Vorontsov et al., 1990) and assure uniform suspension of the organisms during cultivation at 37°C. This determinant confers resistance to uptake by certain professional phagocytes, especially neutrophils and monocytes, but it evidently is ineffective against fixed macrophages lining the capillary network of liver and spleen. This limited ability to protect against phagocytosis in vivo explains, in part, why mutation to Caf1<sup>-</sup> delays, but does not decrease, lethality in the mouse, although a 100-fold reduction in the guinea-pig 50% lethal dose (LD50) was observed (Burrows, 1963; Burrows and Bacon, 1958). Similarly, Caf1<sup>-</sup> mutants provided by aerosol were fully virulent in African green monkeys (Davis et al., 1996), and Caf1-deficient but otherwise typical plague bacilli have been isolated at human autopsy (Winter et al., 1960). Functions other than protection against phagocytosis have been proposed including roles in adhesion. It is interesting in this context that the periplasmic Caf1 chaperone (Caf1M) belongs to a family also involved in assembly of simple surface adhesions as opposed

to FGS chaperones concerned with assembly of complex adhesins such as pili (Chapman et al., 1999). Furthermore, Caf1 was found to exhibit sequence homology and three-dimensional similarity with the interleukin-1 receptor antagonist IL-1ra. Nevertheless, the antigen did not express significant IL-1ra activity or otherwise downregulate proinflammatory cytokines (Krakauer and Heath, 1998).

Murine toxin has been viewed as a significant virulence effector since its discovery by Ramon et al. (1947). As judged by the sequence of its cloned structural gene, native murine toxin probably exists as a 244-kDa tetramer of a 61-kDa primary translational product (Cherepanov et al., 1991). This protein is not toxic in many hosts including guinea pigs and tested primates. However, purified preparations of differing subunit composition have yielded LD50's of 0.2 to 3.7  $\mu$ g in laboratory mice (Perry and Fetherston, 1997). As a consequence, this protein probably is instrumental in causing the prompt death of *Muridae* (Old World rats and mice) during epidemics, thereby assuring the dispersal of infected fleas. Intensive early work on the ability of murine toxin to selectively kill *Muridae* has been reviewed (Brubaker, 1972; Perry and Fetherston, 1997). Resolution of the correct mode of action, however, awaited the results of a BLAST search demonstrating significant homology with phospholipase D (Perry and Fetherston, 1997); murine toxin is now established as a prototype member of the phospholipase D superfamily (Rudolph et al., 1999). A second, and possibly primary, role of murine toxin in facilitating survival in the flea is discussed above.

### pPCP-Encoded Functions

Of the three structural genes present in pPCP, only *pla* encodes an effector of virulence (Sodeinde and Goguen, 1988a); elimination of this function, like loss of *ymt* or *lcrV*, has profound pleiotropic consequences. A particulate fibrinolysin (Pla) discovered by Madison (1936) was later found to activate plasminogen (Beesley et al., 1967). Fractionation of yersiniae resulted in recognition of two forms of the protein in the outer membrane termed  $\alpha$ - and  $\beta$ -Pla (Straley and Brubaker, 1982). Specific message is translated as a transient 34.6-kDa protein that is processed upon insertion into the outer membrane to yield 32.6-kDa  $\alpha$ -Pla. The latter is then converted, probably by shearing, in  $\sim$ 2 hours to slightly smaller  $\beta$ -Pla (Mehigh et al., 1989; Sodeinde and Goguen, 1988a). Both forms bound tenaciously to the outer membrane of *Y. pestis*. However, cells of *E. coli* transformed with pPCP produced only  $\alpha$ -Pla that could be readily extracted in soluble form with 1 M NaCl as a

mixture of  $\alpha$ -Pla, sheared  $\alpha$ -Pla, and smaller forms (Kutyrev et al., 1999). The reason for this discrepancy between yersiniae and *E. coli* is not fully understood, but Pla possesses significant homology with OmpT from the latter (Sodeinde and Goguen, 1989) and may therefore undergo faulty anchoring to the outer membrane of some isolates of this species. Invasion of tissues is accomplished by Pla-dependent adherence to host basement membrane and extracellular matrix, where plasminogen activation facilitates bacterial metastasis (Lähteenmäki et al., 1998). Isolates (pPCP<sup>+</sup>) of *Y. pestis* also can activate prothrombin and thereby coagulate mammalian plasma (Beesley et al., 1967; Eisler, 1961). This function also is accomplished by Pla (Sodeinde and Goguen, 1988a; Sodeinde et al., 1988b). Despite the suggestion that coagulase activity is of limited importance (Sodeinde et al., 1992), the capability may have serious consequences during terminal disease (Eisler, 1961; Finegold et al., 1968).

Expression of Pla is associated with the marked ability of *Y. pestis* to colonize the viscera and thus cause lethal infection upon administration by peripheral (i.e., intradermal, subcutaneous or intraperitoneal) routes of infection. The determinant is not required to initiate lethal disease by intravenous injection, which provides immediate access to fixed macrophages lining the capillary beds of liver and spleen (Brubaker et al., 1965). This relationship, illustrated in Table 3, was verified with isogenic *pla* mutants of epidemic *Y. pestis* strains KIM (Sodeinde et al., 1992) and CO92 (Welkos et al., 1997). Nevertheless, equivalent changes in virulence were not detected in Pla-deficient isolates of Pestoides isolates (Samoilova et al., 1996; Welkos et al., 1997); further studies will be required to determine if the latter possess an additional tissue invasin.

### Chromosome-Encoded Functions

Early studies with *Y. pestis* were instrumental in defining the general importance to pathogenic bacteria of possessing mechanisms capable of high-energy assimilation of iron in vivo. High frequency deletion of the entire *pgm* locus significantly reduces virulence by the intraperitoneal (Jackson and Burrows, 1956a) but not intravenous route of injection (Une and Brubaker, 1984b). It was recognized at the outset that this mutation causes a lesion in ability of *Y. pestis* to assimilate iron because concomitant injection of sufficient Fe<sup>3+</sup> to saturate serum transferrin phenotypically restored full virulence (Jackson and Burrows, 1956b). However, Pgm<sup>-</sup> yersiniae grow well in marginally iron-deficient media, thus verification of a Pgm<sup>+</sup>-dependent lesion in iron transport requires introduction of improved

Table 3. Influence of phenotypic traits on 50% lethal doses of *Yersinia pestis* and *Yersinia pseudotuberculosis* by intravenous, intraperitoneal and subcutaneous routes of injection in control mice and in mice injected with sufficient iron to saturate serum transferrin.

Phenotype			Injected Fe <sup>3+</sup> (40µg/mouse) <sup>a</sup>	Route of injection		
pCD	pPCP	Pgm		Intravenous	Intraperitoneal	Subcutaneous
				LD <sub>50</sub>		
<i>Yersinia pestis</i>						
+	+	+	n	8.1 × 10 <sup>0</sup>	9.9 × 10 <sup>0</sup>	6.1 × 10 <sup>0</sup>
+	+	+	y	9.8 × 10 <sup>0</sup>	9.8 × 10 <sup>0</sup>	5.9 × 10 <sup>0</sup>
-	+	+	n	>5 × 10 <sup>7</sup>	>5 × 10 <sup>7</sup>	>5 × 10 <sup>7</sup>
-	+	+	y	>5 × 10 <sup>7</sup>	>5 × 10 <sup>7</sup>	>5 × 10 <sup>7</sup>
+	0	+	n	7.1 × 10 <sup>1</sup>	3.8 × 10 <sup>5</sup>	>5 × 10 <sup>7</sup>
+	0	+	y	2.3 × 10 <sup>1</sup>	1.4 × 10 <sup>1</sup>	>5 × 10 <sup>7</sup>
+	+	0	n	1.5 × 10 <sup>1</sup>	>5 × 10 <sup>7</sup>	>5 × 10 <sup>7</sup>
+	+	0	y	4.3 × 10 <sup>1</sup>	2.4 × 10 <sup>1</sup>	N.C. <sup>b</sup>
<i>Yersinia pseudotuberculosis</i>						
	pYV <sup>+</sup>		n	3.9 × 10 <sup>1</sup>	2.9 × 10 <sup>4</sup>	1.1 × 10 <sup>4</sup>
	pYV <sup>+</sup>		y	3.1 × 10 <sup>1</sup>	2.0 × 10 <sup>2</sup>	1.4 × 10 <sup>2</sup>
	pYV <sup>-</sup>		n	>5 × 10 <sup>7</sup>	>5 × 10 <sup>7</sup>	>5 × 10 <sup>7</sup>
	pYV <sup>-</sup>		y	>5 × 10 <sup>7</sup>	>5 × 10 <sup>7</sup>	>5 × 10 <sup>7</sup>

Symbols: pCD, pestis virulence plasmid; pPCP, pesticin-producing plasmid; Pgm, pigmentation phenotype; LD<sub>50</sub>, 50% lethal dose; +, phenotype positive; -, phenotype negative; 0, phenotype absent; y, injected; n, not injected; pYV, enterocolitica virulence plasmid.

<sup>a</sup>Iron was administered intraperitoneally as Fe<sup>2+</sup>.

<sup>b</sup>Not calculated.

Summary of data from: Brubaker (1965); Mehig et al. (1989); Une and Brubaker (1984).

methods to remove trace levels of the cation. Curiously, multiplication at 26°C was not inhibited in this environment. Attempts to resolve this issue have now established that plague bacilli utilize at least three chromosomally encoded high-affinity mechanisms to scavenge Fe<sup>3+</sup> in vivo. These processes consist of the siderophore (yersiniabactin)-dependent process encoded by the high-pathogenicity island within the *pgm* locus (Bearden et al., 1997; Buchrieser et al., 1998b; Guilvout et al., 1993; Heesemann, 1987; Rakin et al., 1994), a siderophore-independent yersiniae ferric uptake (Yfu) system (Braun, 1997; Perry, personal communication, 1999), and a siderophore-independent ABC transporter (Yfe) system (Bearden et al., 1998). An additional ill-defined mechanism also may function during growth at 26°C but not 37°C (Lucier et al., 1996; Sikkema and Brubaker, 1987). Furthermore, distinct genes encode products enabling cells of *Y. pestis* to directly utilize hemin and hemoproteins as nutritional sources of iron (Hornung et al., 1996). Loss of this system for assimilating organic forms of iron does not reduce virulence, at least in the mouse (Thompson et al., 1999).

Discovery of two high-molecular-weight proteins termed HMWP1 and HMWP2 (Carniel et al., 1989; Guilvout et al., 1993) provided the basis for establishing the ribosome-independent mechanism of yersiniabactin synthesis. Yersiniabactin is a member of a new class of siderophores

that generally have a lower affinity for Fe<sup>3+</sup> (yersiniabactin  $K_D = 4 \times 10^{36}$ ) than environmental siderophores such as enterochelin ( $K_D = 10^{52}$ ; Gehring et al., 1998); indeed, yersiniabactin was not detected by methods used to determine the stronger ligands (Perry and Brubaker, 1979). Biosynthesis is Fur-regulated (Staggs and Perry, 1992) and constitutes a process dependent upon eleven genes comprising regulatory (*ybtA*), synthetic (*irp2-ybtE*), and transport (*ybtP-ybtS*) operons (Gehring et al., 1998). The structural gene for the unprocessed form of the outer-membrane yersiniabactin receptor (*psn*) maps in this sequence, whereas *ybtP* and *ybtQ* encode inner-membrane permeases required for manipulation of the charged complex. Construction of yersiniabactin involves modular passage of covalent intermediates from the amino terminus of the *irp2* product (HMWP2) to the carboxyl terminus of the *irp1* product (HMWP1). The protein YbtX probably maintains tertiary structure as judged by its hydrophobicity and transmembrane domains. The proteins YbtS, YbtT and YbtE facilitate salicylate, thioesterase and salicyl-AMP metabolism, and YbtA is an AraC-like regulator that promotes transcriptional activation of *psn*, *irp2* and *ybtP*. S-Adenosylmethionine and malonyl-CoA provide the methyl moieties and linker between the thiazoline and thiazolidine rings, respectively. The role of YbtU is unknown but this protein is necessary for biosynthesis (Perry, personal communication, 1999). Pgm<sup>-</sup> mutants

(lacking the entire 102-kb locus) are mimicked with respect to avirulence by isolates possessing point mutations within salient genes comprising the yersiniabactin high-pathogenicity island (Bearden et al., 1997). This finding suggests that the ability to express and utilize yersiniabactin per se accounts for the Pgm<sup>+</sup>-dependent capability of *Y. pestis* to invade the viscera from peripheral sites of infection. Nevertheless, genes encoding putative virulence factors have been mapped between the *hms* locus and high-pathogenicity island that also may contribute to invasiveness (Buchrieser et al., 1999).

The reason why yersiniabactin is dispensable following infection via intravenous injection is not fully resolved. One likely possibility is that host-cell cytoplasm, potentially rich in ferritin-bound and other forms of organic iron, is promptly accessed following intravenous injection. It is therefore significant that mutational loss of the siderophore-independent Yfe or ABC transporter system by Pgm<sup>-</sup> yersiniae (unable to synthesize yersiniabactin) resulted in absolute avirulence (LD<sub>50</sub> <10<sup>7</sup> bacteria) by all routes of injection (Bearden and Perry, 1999). Virulence of mutants cured of pPCP, like those lacking the *pgm* locus, could be restored upon peripheral injection by administration of sufficient iron to saturate serum transferrin (Brubaker et al., 1965). This finding, shown in Table 3, was unexpected because loss of pPCP does not influence the ability of yersiniae to assimilate Fe<sup>3+</sup>. It is now recognized that injected (but not necessarily ingested) iron can inhibit a number of nonspecific mechanisms of host defense (van Asbeck and Verhoef, 1983) and that this situation probably accounted for the ability of iron to enhance the invasiveness of Pla<sup>-</sup> mutants. Indeed, injected iron even restored the virulence of a mutant lacking otherwise essential pCD-encoded cytotoxic YopE (Mehigh et al., 1989).

Cells of *Y. pestis* express at least two additional chromosomally encoded factors that are associated with pathogenicity and shared with *Y. pseudotuberculosis* but not *Y. enterocolitica*. These determinants comprise two of the three temperature-dependent antigens of Crumpton and Davies (1956). The first such activity was designated antigen 3 and is equated with pMT-encoded Caf1. Remaining antigens 4 and 5 are now termed Psa (pH 6 antigen) and KatY, respectively. The reference to pH 6 refers to the penchant of this outer membrane adhesin to undergo optimal synthesis at slightly acidic pH (Bichowsky-Slomnicki and Ben-Efraim, 1963). Mutational loss of pH 6 antigen results in only modest reduction of virulence (Lindler et al., 1990); the minimum target determinant required for binding is β1-linked galactosyl residues in glycosphingolipids (Payne et al., 1998). Some

Psa<sup>-</sup> mutants, like those unable to express Caf1, tend to undergo autoagglutination after growth at 37°C (Crumpton and Davies, 1956). The specific activity of catalase-peroxidase in *Y. pestis* is among the highest reported in prokaryotes (Burrows et al., 1964). This value is due in part to production of a major protein now termed “KatY,” which has marked homology with KatP, a virulence plasmid-encoded catalase-peroxidase of enterohemorrhagic *E. coli* O157:H7 (Garcia et al., 1999a). The consequences of mutational loss of KatY on virulence have not yet been determined but, in view of its abundant temperature-conditional synthesis, it seems possible that the enzyme facilitates expression of disease. One role for KatY is scavenging hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) generated by professional phagocytes, and thereby defeating oxygen-dependent processes of killing. If this mechanism can be verified, further work will be required to determine whether the high catalase activity of plague bacilli contributes to initial survival in fixed macrophages of liver and spleen or in free phagocytic cells encountered during the journey from dermis to viscera. The protein KatY also may function in vivo to degrade H<sub>2</sub>O<sub>2</sub> or organic peroxides generated as terminal electron acceptors in oxygen-deficient niches. It is of interest that KatY, like Yops, is at least partially hydrolyzed by Pla suggesting a transient association with the outer membrane (Garcia et al., 1999b).

REGULATION OF VIRULENCE Shared pCD/pYV encodes an “anti-host genome” (Cornelis et al., 1998b) capable of mediating destruction of mammalian tissue while maintaining the deception that tissue necrosis is under control and the healing process may commence. The type III secretion machine encoded on this plasmid is the engine of virulence in all yersiniae pathogenic to mammals (Cornelis, 1998a; Cornelis et al., 1998b). The localization and description of open reading frames (ORFs) and noncoding elements of pCD1 of *Y. pestis* KIM are shown in Table 4. Many of these genes were initially thought to encode regulatory (*lcr*), type III secretion (*ysc*), virulence effector (*yop*), or chaperone (*ycs*) functions. The original epithets have often been retained in cases where the product was later found to fulfill some distinct or bifunctional role. The ability of plague bacilli to undergo bacteriostasis in vitro in Ca<sup>2+</sup>-deficient media while expressing the pCD-mediated type III secretion system and attendant effectors of virulence is termed “the low-calcium response” or LCR (Goguen et al., 1984). Higuchi and Smith (1961) found that avirulent Ca<sup>2+</sup>-independent mutants are selected at 37°C at a rate of 10<sup>-4</sup> on a medium containing magnesium oxalate; the majority of these mutants are now known to have lost pCD.

Table 4. Localization and description of open reading frames and noncoding elements in pCD1.

ORF or non-coding element	Position no.	Size		Description		
		Amino acids	Mass (kDa)	Orientation	Gene	Function
				ORFs		
1	87–1109	340		+		Transposase
2	1109–1899	259		+		Transposase
3	1939–2343	134		–		Transposase
4	2379–2645	88		–	<i>lcrS</i>	
5	3193–3540	115	12.41	–	<i>lcrQ (yscM)</i>	Negative regulator of LCS
6	3765–4430	221	24.65	–	<i>yscL</i>	Type III secretion component
7	4376–5005	209	23.99	–	<i>yscK</i>	Type III secretion component
8	5005–5739	244	27.04	–	<i>yscJ</i>	Type III secretion component
9	5746–6093	115	12.67	–	<i>yscI</i>	Type III secretion component
10	6094–6591	165	18.35	–	<i>yscH (yopR)</i>	Secreted; unknown function
11	6588–6935	115	13.07	–	<i>yscG</i>	Type III secretion component
12	6937–7200	87	9.49	–	<i>yscF</i>	Type III secretion component
13	7201–7401	66	7.61	–	<i>yscE</i>	Type III secretion component
14	7398–8657	419	46.93	–	<i>yscD</i>	Type III secretion component
15	8654–10477	607	67.35	–	<i>yscC</i>	Type III secretion component
16a	10483–10896	137	15.41	–	<i>yscB</i>	Unknown
16b	11121–11220	32	3.86	–	<i>yscA</i>	Unknown
17	11299–12114	271	30.84	–	<i>lcrF (virF)</i>	Transcriptional activator
18	12238–12633	131	14.71	–	<i>yscW (virG)</i>	YscC chaperone
19	13209–14273	354	40.39	–	<i>yscU</i>	Type III secretion component
20	14273–15058	261	28.45	–	<i>yscT</i>	Type III secretion component
21	15055–15321	87	9.57	–	<i>yscS</i>	Type III secretion component
22	15323–15976	217	24.43	–	<i>yscR</i>	Type III secretion component
23	15973–16896	307	34.42	–	<i>yscQ</i>	Type III secretion component
24	16893–18260	455	50.42	–	<i>yscP</i>	Type III secretion component
25	18260–18724	154	19.00	–	<i>yscO</i>	Type III secretion component
26	18721–20040	439	47.81	–	<i>yscN</i>	Type III secretion component
27	20238–21119	293	32.67	+	<i>lcrE (yopN)</i>	Ca <sup>2+</sup> sensor; secretion control
28	21100–21378	92	10.75	+	<i>tyeA</i>	Yop targeting and secretion
29	21365–21736	123	13.61	+	ORF	Unknown
30	21733–22101	122	13.76	+	ORF	Unknown
31	22098–22442	114	13.21	+	ORF	Unknown
32	22429–24543	704	77.81	+	<i>lcrD (yscV)</i>	Yop secretion
33	24540–24980	144	16.46	+	<i>lcrR</i>	Unknown (Ca <sup>2+</sup> -blindness)
34	25022–25309	95	11.02	+	<i>lcrG</i>	Yop and LcrV translocation
35	25311–26291	326	37.24	+	<i>lcrV</i>	Yop secretion and control; anti-inflammatory effector
36	26304–26810	168	19.02	+	<i>lcrH (sycD)</i>	YopB and YopD chaperone
37	26788–27993	401	41.83	+	<i>yopB</i>	Yop targeting
38	28012–28932	306	33.39	+	<i>yopD</i>	Yop targeting; negative control
39	29345–29512	55		–	ORF	Unknown
40	29778–30038	87		–		Transposase
41	30873–32102	409	46.21	+	<i>yopM</i>	Virulence effector
42	32145–32444	99		–		Transposase
43	34860–35828	322	36.21	–	<i>yopT</i>	Virulence effector
44	36328–36876	182	21.00	+	<i>yopK (yopQ)</i>	Yop targeting
45/46	37360–38110	36		+	<i>(ylpA)</i>	(Pseudogene)
47	38624–39016	130		+		Transposase
48	40080–41288	402		+	<i>sopA</i>	Plasmid partitioning
49	41417–42250	277		+	<i>sopB</i>	Plasmid partitioning
50	44186–44845	219	22.99	–	<i>yopE</i>	Virulence effector
51	45039–45431	130	14.65	+	<i>sycE</i>	YopE chaperone
52	45494–46123	309		–		Transposase
53	46241–47413	390		+		Transposase
54	47413–47844	143		+		Transposase
55	48188–48613	141	15.76	+	<i>sycH</i>	YopH chaperone
56	49594–49860	88		–		Transposase
57	50911–51462	183		–		Transposase ( <i>tmpR</i> )
58	51626–53941	771		+		Transposase ( <i>tmpA</i> )

Table 4. *Continued*

ORF or non-coding element	Position no.	Size		Description		
		Amino acids	Mass (kDa)	Orientation	Gene	Function
59	53938–54318	206		–		Transposase
60/61	54924–56227	50		+	( <i>yadA</i> )	(Pseudogene)
62	56488–56297	63		+	ORF	Unknown
63	56928–57344	138		+		DNA helicase I (F plasmid)
64	58681–58929	82		+		Endonuclease
65	59067–59321	84	9.58	+	<i>repB</i>	Negative regulator of <i>repA</i>
66	59618–60496	292	33.55	+	<i>repA</i>	Plasmid replication
67	63100–65298	732	81.74	+	<i>ypkA (yopO)</i>	Virulence effector
68	65694–66557	287	32.46	+	<i>yopJ (yopP)</i>	Virulence effector
69	67146–67649	167		–		Transposase
70	68243–69649	468	50.87	+	<i>yopH</i>	Virulence effector
71	70502–70161	113		–		Partial transposase
Noncoding elements						
	1–1954			+		IS100
	2655–1961			–		ISD1
	5716–4973			–		IS285
	29501–30801			+		R1 repeat of <i>yopM</i>
	32118–34817			+		R2 and R3 repeat of <i>yopM</i>
	36952–37179			+		IS1351 and IS200
	38717–39225			+		IS911
	43032–43399			+		IS1327
	46137–47226			+		IS640
	46137–45463			–		<i>nifJ</i> /IS2222
	49755–49521			–		ISR1
	48647–48728			+		IS285 (partial)
	47600–47786			+		IS100 (partial)
	53976–52230			–		Tn3 homolog (partial)
	60005–61324			+		R100 maintenance region
	70001–70145			–		IS285 (partial)
	70504–70322					IS1351 and IS200

Symbols: ORF, open reading frame; “+”, “–” refers to orientation.

Data from Hu et al. (1998) and Perry et al. (1998).

However, point mutations on pCD of similar phenotype also can be selected with this medium. These mutants, like isolates cured of pCD, are not only Ca<sup>2+</sup>-independent but also lack expression of the type III secretion mechanism and attendant virulence factors. This type of mutant was instrumental in defining the regulation and physiology of type III secretion. Paramount among Ca<sup>2+</sup>-independent point mutations is *lcrF* noted above, which encodes a general transcriptional activator of pCD-encoded functions (Cornelis et al., 1989; Cornelis, 1993; Hoe and Goguen, 1993; Lambert de Rouvroit et al., 1992; Skurnik and Toivanen, 1992; Wattiau and Cornelis, 1994) and acts as the thermostat recognizing host temperature (Rohde et al., 1999).

Another example is *lcrV*, encoded within the *lcrGVH-yopBD* operon (Bergman et al., 1991; Perry et al., 1986; Price et al., 1989b). A multifunctional protein (LcrV; Skrzypek and Straley, 1995) was initially implicated as both a regulator of the LCR (Bergman et al., 1991; Price et al., 1991) and a virulence factor (see Disease in this

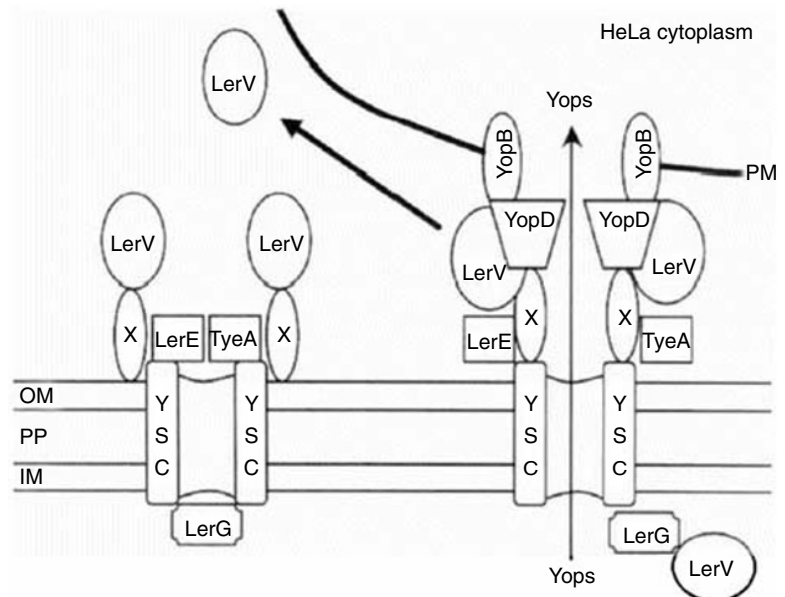
Chapter). However, evidence has not appeared as yet demonstrating a classical role for LcrV in DNA binding or other traditional forms of transcriptional control. The mechanism whereby LcrV upregulates other LcrF-activated gene products is not fully resolved but its export from the bacterium was found to depend upon interaction with genetically linked LcrG (Nilles et al., 1997). This observation is consistent with the prospect that LcrV removes excess LcrG by titration. It is, therefore, the latter that actually functions to downregulate LcrF-activated functions. The finding that LcrG serves to facilitate excretion of LcrV was extended by Sarker et al. (1998a), who reported that LcrG also is required for mobilization of cytotoxic Yops during their translocation. Excellent evidence has now accrued indicating that LcrV is an integral component of the type III secretion machine where it serves to facilitate translocation of effector Yops or extrusion of other components of the translocation apparatus (Fields et al., 1999b; Nilles et al., 1998; Pettersson et al., 1999; Sarker

et al., 1998b). Nevertheless, export of LcrV itself is not dependent upon the pCD-encoded secretion machine as demonstrated by its translocation by an independent chromosomally encoded type III secretion system (Fields and Straley, 1999a). Additional  $\text{Ca}^{2+}$ -independent variants exist including *lcrD* encoding an inner-membrane core-secretion function (Plano and Straley, 1981). So-called “ $\text{Ca}^{2+}$ -blind” mutants constitute a second phenotype (Goguen et al., 1984) that has been equally instrumental in defining the LCR. These isolates are temperature-sensitive in that they are unable to sustain vegetative growth at  $37^\circ\text{C}$  even in the presence of sufficient  $\text{Ca}^{2+}$  to assure multiplication of the parent. In addition,  $\text{Ca}^{2+}$  is generally unable to downregulate LcrF-activated genes in these mutants, although thermoregulation of LcrF-activated gene products remains normal. As might be expected from the above, *lcrG* is a  $\text{Ca}^{2+}$ -blind mutant (Boyd et al., 1998; Skrzypek and Straley, 1993) by virtue of its inability to prevent accumulation of internal LcrF-activated gene products. This phenotype also is observed upon mutational loss of LcrE (YopN), which functions as a sensor of external  $\text{Ca}^{2+}$  (Forsberg et al., 1991). Other  $\text{Ca}^{2+}$ -blind mutants are *lcrR* (Barve and Straley, 1990) and both *yopD* (Håkansson et al., 1993) and its *lcrH* (*syncD*) chaperone (Bergman et al., 1991; Price and Straley, 1989a).

The YopD in concert with YopB and other pCD-encoded proteins noted below, initiate the type III secretion process used for translocation of cytotoxic Yops. The physiology of this process is illustrated in Fig. 4 from Fields et al. (1999b). The expression of virulence effectors translocated by the secretion machine is downregulated

by  $\text{Ca}^{2+}$ , which indirectly prevents their secretion, thereby promoting a form of negative feedback inhibition. Either removal of  $\text{Ca}^{2+}$  from the environment (especially in excess  $\text{Mg}^{2+}$ ) or intimate contact with host cells in the presence of  $\text{Ca}^{2+}$  eliminates this block, whereupon LcrF-regulated determinants undergo prompt synthesis and export. Calcium may downregulate their exit in the absence of host cell contact by stabilizing an outer gate block of translocation channels mediated by LcrE (Forsberg et al., 1991) and TyeA (Iriarte et al., 1998b). Removal of the block by either withdrawal of  $\text{Ca}^{2+}$  or docking to host cells promotes dissipation of negative control upon secretion of the negative regulator LcrQ and YopD (Williams and Straley, 1998). The latter, in concert with YopB and LcrV, then forms a translocation pore modulated by YopK (Holmström et al., 1997). It is through this pore (raised from the basic secretion core foundation) that pCD-encoded cytotoxic Yops are translocated to targets located within host cell cytoplasm. Removal of the LcrG-mediated impediment by titration with internal LcrV clears the passage through which effector Yops enter the host cell (Nilles et al., 1998). The LcrV protein thus functions as both a structural component of the outer portion of the injection apparatus and as a cytoplasmic element required for retraction of LcrG from the inner gate of the secretion pore. As noted above, LcrV also is translocated to host cell cytoplasm via a separate process mediated by chromosomal genes (Fields and Straley, 1999a). As a consequence of this process, as well as its dissipation from the injection apparatus, considerable extracellular LcrV accumulates *in vitro* (Lawton et al., 1963) and *in vivo* (Smith et al., 1960).

Fig. 4. Model for the role of surface-located pCD-encoded proteins in mediating translocation of effector Yops. The Ysc creates a secretion channel through the bacterial inner membrane (IM), across the periplasm (PP), and through the outer membrane (OM). These pores remain blocked in the absence of direct contact with the eukaryotic cell plasma membrane (PM) by LcrE, TyeA and LcrG. The LcrV protein is associated with the extracellular surface of these closed pores directly or by association with another protein (X). Upon host cell contact, LcrE, TyeA and LcrG are displaced, and LcrV mediates formation of a delivery apparatus for transfer of Yops directly from the bacterium to the HeLa cell (eukaryotic) cytoplasm. (From Fields et al., 1999b).



The injection apparatus shown in Fig. 4 may exist in a dynamic state undergoing continuous reassembly (Fields et al., 1999b). Release of free YopB and YopD (like LcrV) during this process might therefore contribute to the overall lytic effect caused by delivery of the total package of cytotoxic Yops to host target cells. Tacit to these observations is the understanding that normal upregulation of at least one LcrF-activated gene product results in an as yet unknown bioenergetic drain that eventually promotes bacteriostasis *in vitro*. As noted above, this induction is normally prevented at room temperature by maintenance of inactive *lcrF* superstructure (Rohde et al., 1999) and at 37°C by Ca<sup>2+</sup>-YopN interaction (Forsberg et al., 1991). Further study will be required to fully resolve the mechanism whereby Ca<sup>2+</sup> prevents bacteriostasis at 37°C, but it is interesting that this cation is not required for full-scale growth upon removal of Na<sup>+</sup> and L-glutamate (Fowler and Brubaker, 1994). It is significant in this context that otherwise Ca<sup>2+</sup>-blind *lcrE*, *lcrR*, and *lcrG* mutants, like the wild-type parent, express LcrF-activated genes and exhibit nearly full-scale growth in medium lacking both added Ca<sup>2+</sup> and Na<sup>+</sup> (R. Brubaker, unpublished observations). However, neither *yopD* mutants nor isolates unable to express the YopD chaperone (*lcrH*) could multiply at 37°C in this environment. The temperature-sensitivity of the *yopD* mutant could, however, be fully suppressed by introduction of *lcrD*. These findings are consistent with the occurrence of two mechanisms of bacteriostasis. The first, as shown in Fig. 2, results from Na<sup>+</sup> toxicity to yersiniae expressing LcrF-mediated functions and may relate in part to loss of metabolic carbon. The second rests on the inability to produce or extrude YopD and may reflect the occurrence of physical damage caused by failure to construct a functional secretion machine.

This secretion process shown in Fig. 4 is distinct in the enteropathogenic yersiniae. Undelivered Yops produced by the latter, such as those expressed during cultivation *in vitro* at 37°C in Ca<sup>2+</sup>-deficient media, are released into the supernatant fluid, become denatured, and nonspecifically absorb to the bacterial outer membrane as well as the culture vessels (Michiels et al., 1990). In contrast, undelivered Yops similarly produced by cells of *Y. pestis* harboring pPCP do not accumulate but rather undergo immediate posttranslational degradation (Fig. 5). The assumption that this phenomenon is mediated by Pla rather than pesticin or its immunity protein (Sample and Brubaker, 1987a; Sample et al., 1987b) was verified by genetic analysis (Sodeinde et al., 1988b). Transfer of pPCP to *Y. pseudotuberculosis* harboring pYV also prompted posttranslational degradation of Yops, although only partial

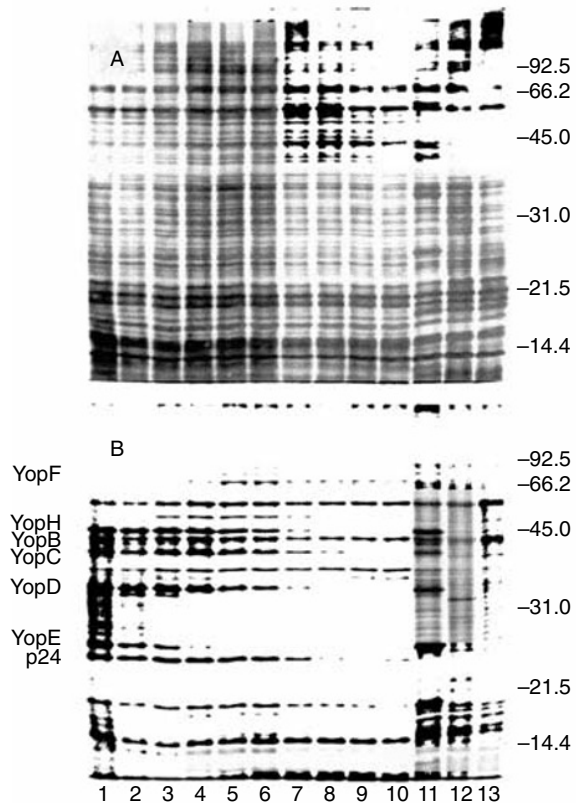


Fig. 5. Silver-stained gel (A) and corresponding autoradiogram (B) of trichloroacetic acid-precipitated material from cultures of *Y. pestis* KIM10 cultivated at 37°C in Ca<sup>2+</sup>-deficient medium. Cells (pCD<sup>+</sup>, pPCP<sup>+</sup>) were pulsed for 15 s with <sup>35</sup>S-methionine and then chased with unlabeled methionine for 0 (lane 1), 15 s (lane 2), 30 s (lane 3), 1 min (lane 4), 2 min (lane 5), 4 min (lane 6), 8 min (lane 7), 15 min (lane 8), 30 min (lane 9) and 1 h (lane 10). Control lanes (pulsed for 15 s and then chased for 1 h) contained isogenic pCD<sup>+</sup>, pPCP<sup>+</sup>? (lane 11), pCD<sup>-</sup>, pPCP<sup>+</sup> (lane 12), and pCD<sup>-</sup>, pPCP<sup>-</sup> (lane 13) derivatives. Molecular weight markers in kDa are provided in the right margin and the positions of individual Yops are shown in the left margin; p24 indicates a stable YopE degradation product (Chalvignac et al., 1988). (From Kuttyrev et al., 1999.)

hydrolysis of the adhesin YadA was observed (Kuttyrev et al., 1999). As noted under Disease, undelivered Yops would be recognized as foreign protein and would thus promote inflammation. The ability of *Y. pestis* to eliminate this material from its immediate vicinity during growth in necrotic visceral lesions could provide another parameter of stealth contributing to the acute symptoms of plague.

It is now evident that bulk vegetative protein is not synthesized by pCD<sup>+</sup> yersiniae under conditions of strict bacteriostasis imposed by the LCR, although marked expression of Yops and LcrV occurs in Ca<sup>2+</sup>-deficient media. This relationship is consistent with the possibility that at least one LcrF-activated function promotes an as



yet undefined lesion in bioenergetics accounting for bacteriostasis. Virulence effectors encoded on the chromosome, pMT and pPCP also are produced in this environment during bacteriostasis including Caf1, Psa, KatY, Pla and murine toxin (as well as the ubiquitous chaperone GroEL; Mehigh and Brubaker, 1993). This finding suggests that these activities may be regulated by mechanisms that are independent of those known to control transcription of proteins required for generation of cell mass. It may, therefore, be significant in this context that the KatY promoter contained three homologs to the consensus recognition sequence of pCD-encoded transcriptional activator LcrF (Garcia et al., 1999a). Despite the presence of these sequences, synthesis of KatY remains temperature-conditional in mutants lacking pCD (Crumpton and Davies, 1956), suggesting that a second LcrF-like transcriptional activator is encoded within the chromosome.

## Genetics

### Historical

The modern era of research on the genetics of virulence in yersiniae commenced in 1980 upon the discovery of pYV by (Zink et al., 1980). Nevertheless, the experimental system developed earlier by T. W. Burrows and colleagues first illustrated the utility of defining virulence factors on the basis of mutant phenotype (Burrows, 1960; Burrows, 1963; Burrows and Bacon, 1958). The modification of experimental methods suitable for genetic analysis of yersiniae has kept pace of those techniques developed for other members of the Enterobacteriaceae. Shared methods include the use of certain conjugative plasmids or bacteriophages that are unable to integrate or replicate in yersiniae (i.e., the sex factor F<sup>+</sup>, P1 and  $\lambda$  for mediating gene transfer). Protocols are available for induction and selection of auxotrophic mutants (Brubaker, 1970a). Studies employing standard methods of transposon and bacteriophage insertion mutagenesis have been instrumental in identifying genes encoding virulence effectors and regulators. Use of these techniques has provided assurance that plague bacilli possess typical mechanisms of DNA repair as judged by UV induction of the bacteriocin pesticin (Ben-Gurion and Hertman, 1958; Hu et al., 1972), effectiveness of radiomimetic agents in inducing mutations (Brubaker, 1970b), and isolation of RecA<sup>-</sup> mutants (Hare and McDonough, 1999a). The three plasmids of *Y. pestis* can readily be obtained by standard techniques and transformed by electroporation (Perry and Fether-

ston, 1997). Utilization of ligand-fusion protein engineering has been especially useful in defining the relationship between immunity and repression of proinflammatory cytokines as defined under Disease (Anderson et al., 1996; Fields and Straley, 1999a; Leary et al., 1995; Motin et al., 1994; Motin et al., 1996; Schmidt et al., 1999; Welkos et al., 1998).

### The Genome

A genome of 4,208.4 kb was determined for *Y. pestis* strain KIM10 by two-dimensional pulsed-field gel electrophoresis of chromosomal DNA first digested with *Spe*I and then *Apa*I (Lucier and Brubaker, 1992). Addition of 9.7, 70.5 and 100.9 kb obtained by sequencing pPCP1, pCD1 and pMT1, respectively (Hu et al., 1998; Lindler et al., 1998; Perry et al., 1998), yields a total genome of 4,389.5 kb. This value is about 90% of that reported for *E. coli*. Extensive regions of the chromosome also have been sequenced (*Yersinia pestis* Sequencing Group, on-line) including the deletable *pgm* locus (Buchrieser et al., 1999) and the region encompassing *katY* (Garcia et al., 1999b). As noted under Phylogeny, almost 0.1% of the total genome consists of up to 50 copies of insertion sequence (IS) elements, which both diminish coding capacity and facilitate deletion as first shown for the *pgm* locus (Fetherston and Perry, 1994; Fetherston et al., 1992).

### The *pgm* Locus

Genetic analysis of the *pgm* locus has provided definitive information regarding the stability of the genome and consequences of reciprocal recombination promoted by proximal IS elements. The possibility that the chromosome undergoes rapid internal rearrangement was raised by Itehan et al. (1993) who detected Hms<sup>-</sup> mutants of laboratory strains that retained *irp2* within the high-pathogenicity island, although the reciprocal was not observed. Loss of the Hms<sup>+</sup> phenotype in these strains was attributed in part to smaller deletions encompassing all or portions of the Hms region or to point mutations therein (Buchrieser et al., 1998b). This observation was inconsistent with the earlier report that the genome of *Y. pestis* is stable as judged by repeatable generation of precise DNA fragments by restriction enzyme digestion, and with the report that high-frequency mutation to Pgm<sup>-</sup> was accompanied without exception by loss of an identical -100-kb sequence (Lucier and Brubaker, 1992). The concept of an unstable genome in *Y. pestis* also is inconsistent with the observations that the *pgm* locus undergoes precise elimination by reciprocal recombination (Fetherston and Perry, 1994; Fetherston et al.,

1992) and that only Pgm<sup>+</sup> isolates (containing *psn*) but lacking pPCP (encoding the pesticin immunity protein) are sensitive to pesticin (Brubaker, 1970a).

Selection for insensitivity to pesticin would be expected to yield rare Psn<sup>-</sup> mutants (unable to assimilate yersiniabactin) entirely analogous to variants of *E. coli* resistant to group B colicins (Davis and Reeves, 1975). Mutation to Pgm<sup>-</sup> does, of course, result in loss of the high-pathogenicity island (encoding the synthesis and assimilation of yersiniabactin) within which *psn* resides (Bearden et al., 1997; Buchrieser et al., 1998b; Buchrieser et al., 1999; Fetherston et al., 1995; Rakin et al., 1994). However, to select for the rare occurrence of point mutations within *psn*, it became necessary to score for insensitivity to pesticin plus retention of the *hms* locus (as judged by ability to autoagglutinate at room temperature). Cells of one such isolate retained the ability to absorb exogenous hemin or Congo red at 26°C. However, like typical Pgm<sup>-</sup> mutants, these cells were unable to sustain growth at 37°C in iron-deficient medium (Sikkema and Brubaker, 1989). Analysis of this variant revealed that it possessed a 5-bp deletion (GACCT) in *psn*, causing premature termination of translation. This sequence occurred immediately after another GACCT run, thus its removal probably reflects a rare error in DNA replication (Lucier et al., 1996).

These findings illustrate that it is difficult to select for the occurrence of individual mutations in genes comprising the *pgm* locus due to spontaneous loss of the locus at high frequency. This point was emphasized by Hare and McDonough (1999a) who verified that pesticin-resistant mutants of a RecA<sup>+</sup> Pgm<sup>+</sup> strain of *Y. pestis* (cured of pPCP) arise at very high frequency and lack the entire *pgm* locus. Arising in an isogenic RecA<sup>-</sup> isolate at a lower frequency, which would prohibit their detection in a RecA<sup>+</sup> background, Hms<sup>-</sup> mutants usually retained at least portions of the high-pathogenicity island. The RecA<sup>-</sup> isolate also yielded rare pesticin-resistant mutants that were typically Hms<sup>+</sup> (Hare and McDonough, 1999a). It is probably significant that the KIM strain was used in these experiments verifying that the high-frequency mutation to Pgm<sup>-</sup> reflects reciprocal recombination between IS elements and that the genome is otherwise stable. As noted by Hare and McDonough (1999a), isolates found to undergo alternative mutational events resulting in high frequency acquisition of the Hms<sup>-</sup> phenotype (Buchrieser et al., 1998b; Iteman et al., 1993) represent laboratory stocks that may have accumulated additional IS elements or homologous sequences within the *pgm* locus during storage. This difference between strain KIM and laboratory stocks emphasizes the necessity of utilizing

isogenic derivatives of virulent isolates in defining parameters of pathogenesis.

## Epidemiology

As noted under Phylogeny, *Y. pestis* is an exception to the epidemiological tenet of Theobald Smith (Smith, 1934) that states it is a flawed strategy for a parasite to unduly damage its host because by so doing that parasite compromises the only environment capable of supporting its existence in nature. The rationale for this anomaly is that death of the current host assures departure of the flea vector in search of a new host, thereby perpetuating plague bacilli in nature. The pathogens comprising this errant group are few, but like *Bacillus anthracis* (which must kill its host to assure efficient conversion to infectious spores), they rank among the deadliest known to humans.

Plague bacilli are newcomers in geological terms (Achtman et al., 1999); thus, insufficient time has elapsed since their emergence as a species to permit extensive clonal divergence. Nevertheless, limited but stable differences in electropherogram polymorphism occur among typical strains (Lucier and Brubaker, 1992) that should provide useful epidemiological markers. Similarly, phenotypic differences in the ability to ferment glycerol and reduce nitrite have proven useful in distinguishing between strains thought to be responsible for the three pandemics of history (Devignat, 1951). Isolates capable of both functions are of the biovar *antiqua*, believed to remain as holdovers from the first pandemic starting with the Justinian plague of the 6th century. Strains only able to ferment glycerol represent the biovar *medievalis* accountable for the second pandemic of Europe initiated during the 14th century by the Black Death. The biovar *orientalis* consists of isolates lacking both activities; these strains are responsible for the third pandemic of modern times. The legitimacy of this schema was underscored by the discovery of other genes encoded by the *pgm* locus that have undergone analogous sequential loss (Buchrieser et al., 1999). Similarly, Popov et al. (1998) demonstrated the occurrence of inversions resulting in differences of 3 kb in size between pMT from strains of the *antiqua* and *orientalis* biovars.

The three biovars noted above represent clones that have escaped confinement within the ancient plague reservoirs of the Russian steppes, central China and Mongolia. Less is known about the genetic makeup of Pestoides isolates or other strains resident to these areas, but local lore pronounces that strains recovered within some locations are attenuated, whereas those

from other regions are of extraordinary virulence. Similarly, variants possessing plasmids of distinct size or number have been isolated within these very old foci where the species probably emerged. One possibility that has received consideration in this context is that plague bacilli native to these regions may possess different genomic configurations than do the epidemic biovars of Devignat (1951). If so, the endemic forms would nevertheless remain capable of reciprocal recombination between IS elements, and thus be able to undergo almost unlimited chromosomal rearrangement via internal transposition and inversion, including return to the epidemic form (accounting for the occurrence of pandemics every 700 years or so). Further study of strains indigenous to the ancient plague reservoirs of central Asia will be required to substantiate this notion of a plastic genome.

## Disease

It is generally trivial to demonstrate by genetic analysis that a given gene or product thereof is necessary for expression of virulence (although study of *Y. pestis* mutants also has repeatedly shown that determinants thought to be critical are dispensable and vice versa). It is altogether another matter to correctly identify how an essential determinant actually mediates disease. Before considering these issues, a description of experimental plague in mice will be provided and compared to the human disease. This exercise will emphasize unique features of the infection and facilitate later assignment of function to known effectors of virulence.

### Pathology of Plague

As noted under Phylogeny, the remarkable ability of wild-type *Y. pestis* to cause lethal disease via peripheral (intradermal, subcutaneous or intraperitoneal) routes of infection is not shared by *Y. pseudotuberculosis* (Brubaker et al., 1965), although both species are of comparable high virulence when injected intravenously (Table 3). This distinction between routes of administration occurs because, as first shown in guinea pigs (Janssen et al., 1958), intravenously injected yersiniae undergo immediate removal from the vascular system upon passage through liver and spleen. This form of clearance is mediated by physical sieving within capillary beds, as well as phagocytosis by fixed macrophages (Janssen et al., 1958). Following filtration from the circulation, both of these facultative intracellular parasites soon emerge within interstitial spaces to form localized extracellular foci of infection (Nakajima et al., 1995; Simonet et al., 1990;

Straley and Cibull, 1989; Une et al., 1986). This sequence does not occur following peripheral administration where the enteropathogenic species are typically immobilized and eliminated at the original site of infection. In contrast, peripherally injected cells of *Y. pestis* possess a unique ability to invade adjacent tissues and eventually access favored niches within the viscera (Brubaker et al., 1965; Sodeinde et al., 1992; Welkos et al., 1997).

The mammalian host responds to bacterial invasion or the insult of injected foreign matter by promptly upregulating major proinflammatory cytokines. Especially important mediators of this generic inflammatory response are IFN- $\gamma$  and TNF- $\alpha$ , known to fill indispensable roles in nonspecifically activating professional phagocytes and mediating formation of protective granulomas. Cells of *Y. pestis* lacking pCD resembled those of *E. coli* or other common bacteria in this regard as judged by their ability to promptly induce IFN- $\gamma$  and TNF- $\alpha$  upon intravenous injection into mice (Fig. 6A). However, intravenously injected yersiniae harboring pCD downregulated expression of IFN- $\gamma$  throughout the course of disease, although some TNF- $\alpha$  was produced after the mice had become moribund (Fig. 6B). This astonishing ability of pCD to abrogate inflammation could be reversed by priming the mice with trace levels of IFN- $\gamma$  plus TNF $\alpha$  before infection (Fig. 6C) or by passive immunization with a polyclonal antiserum raised against pCD-encoded LcrV (Fig. 6D), whereupon the mice survived the infection (Nakajima and Brubaker, 1993). Wild-type cells of *Y. pestis* thus possess the truly remarkable faculty of preventing the generic inflammatory response necessary for eventual development of delayed-type hypersensitivity. This finding illustrates that the ability of *Y. pestis* to cause death does not reflect successful invasion of the host by frontal assault. Instead, lethality reflects an ability to disguise ongoing mortal tissue destruction, thereby maintaining the illusion that the host has no cause for alarm. In short, plague is a disease of stealth.

After accessing the viscera (either indirectly via lymphatic vessels and solid tissue or directly by intravenous injection), pCD<sup>+</sup> yersiniae assume residence and commence multiplication in spleen and liver (Fig. 7). Thereafter, the lungs are invaded and replication progresses until organ function is lost and the mouse becomes moribund. By this time, significant net accumulation of bacteria occurs within the vascular system, thereby assuring infection of resident fleas that now partake of their last blood meal before departing in search of a new host. Intravenously injected yersiniae lacking pCD are similarly filtered from liver and spleen where they initiate growth in a manner similar to pCD<sup>+</sup> organisms.

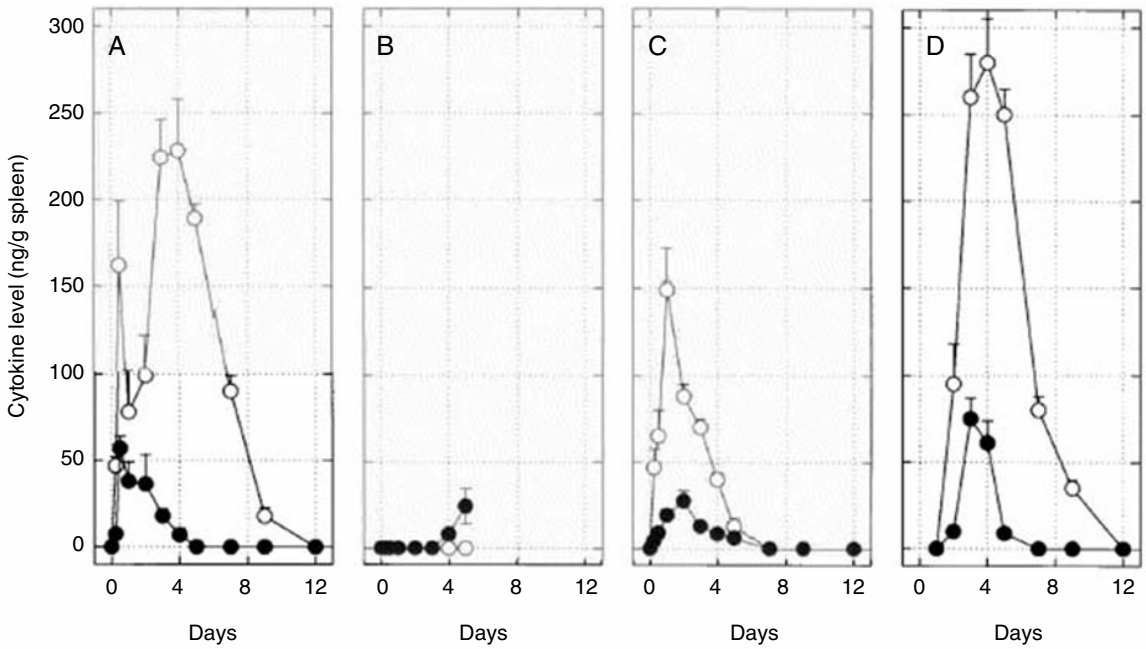


Fig. 6. Expression of the proinflammatory cytokines IFN- $\gamma$  (○) and TNF- $\alpha$  (●) in C57/BL/6 mouse spleen following intravenous challenge with (A),  $10^6$  pCD $^-$  cells of *Y. pestis* KIM10; (B),  $10^2$  pCD $^+$  cells of *Y. pestis* KIM10; (C),  $10^2$  pCD $^+$  cells of *Y. pestis* KIM10 after priming with 20  $\mu$ g of IFN- $\gamma$  and 20 ng of TNF- $\alpha$  □ and (D),  $10^2$  pCD $^+$  cells of *Y. pestis* KIM10 upon passive immunization on postinfection day 1 with 100  $\mu$ g of  $\gamma$  polyclonal rabbit anti-LcrV. All untreated mice challenged with pCD $^-$  yersiniae died by postinfection day 6 (B) whereas the remainder all survived. Redrawn from Nakajima and Brubaker (1993) and Nakajima et al. (1995).

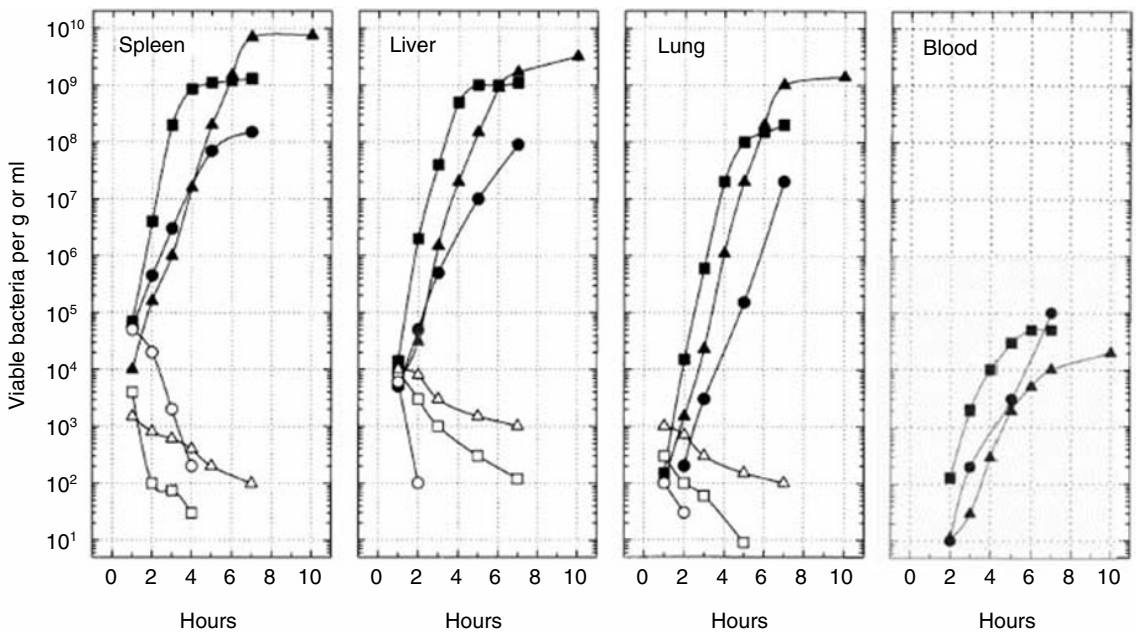


Fig. 7. Growth of pCD $^+$  (●) and pCD $^-$  (○) *Y. pestis* KIM10, pYV $^+$  (■) and pYV $^-$  (□) *Y. pseudotuberculosis* PB1, and pYV $^+$  (▲) and pYV $^-$  (△) *Y. enterocolitica* WA in mouse spleen, liver, lung and blood following intravenous injection of  $10^2$  pCD/pYV $^+$  yersiniae and  $10^4$  pCD/pYV $^-$  mutants (T. Une and R. Brubaker, unpublished observations).

However, proliferation soon ceases within these foci and the bacteria become eliminated without reentering the vascular system (Nakajima et al., 1995; Une et al., 1986).

Predominant extracellular growth of *Y. pseudotuberculosis* in visceral organs was first reported by Simonet et al. (1990). Inspection of thin sections from liver and spleen revealed that pCD<sup>+</sup> isolates of *Y. pestis* also grew extracellularly within enclosed non-vascularized necrotic foci (Nakajima et al., 1995; Une et al., 1986). These necrotic lesions progressively enlarged and then coalesced as the infection continued (Fig. 8A). At no time did the host mount a detectable inflammatory response against the bacteria contained within these foci, which eventually hemorrhaged resulting in spillover into the vascular system. Like the pCD<sup>+</sup> parent, intravenously injected pCD<sup>-</sup> yersiniae also assumed residence within interstitial spaces of visceral organs, but the resulting lesions were almost immediately surrounded by inflammatory cells that promptly facilitated formation of protective granulomas (Fig. 8B). Throughout the course of disease, bulk vegetative growth occurred almost exclusively within spleen, liver and then lung (Fig. 7). These organs became literally consumed and were almost stoichiometrically converted to bacterial mass. Because bulk vegetative growth occurs primarily within cytoplasm released into closed necrotic foci, the ability to resist uptake by neutrophils and monocytes is of minimal

importance as a mechanism preventing acute disease. Reliance upon liver and spleen as favored niches for bulk vegetative growth does not, of course, preclude a role for professional phagocytes in delaying invasion of the viscera following peripheral administration or upon infection of the dermis by fleabite. The terminal population of intravenously injected enteropathogenic yersiniae in moribund mice is typically about ten times that observed for *Y. pestis* which, at comparable challenge doses, also causes an earlier death (Fig. 7). As noted under Physiology, this distinction between *Y. pestis* and *Y. pseudotuberculosis* may reflect, in part, the lethal action of pMT-encoded murine toxin.

The succession of events in human plague is similar to those observed in the mouse and many other rodents, except that these natural hosts usually expire before comparable lymphadenopathy can develop. Typical symptoms of untreated plague in humans include pronounced fever, headache and local edema accompanied by tenderness of the regional lymph nodes draining the extremity receiving the infecting fleabite. These (cervical, femoral/inguinal or axillary) lesions are termed “buboes”; they delay but generally fail to prevent eventual invasion and colonization of the viscera in a manner analogous to that described in the mouse. Indeed, removal of the invading bacteria by filtration within regional lymph nodes may sometimes fail entirely, thereby facilitating bacteremia and visceral inva-

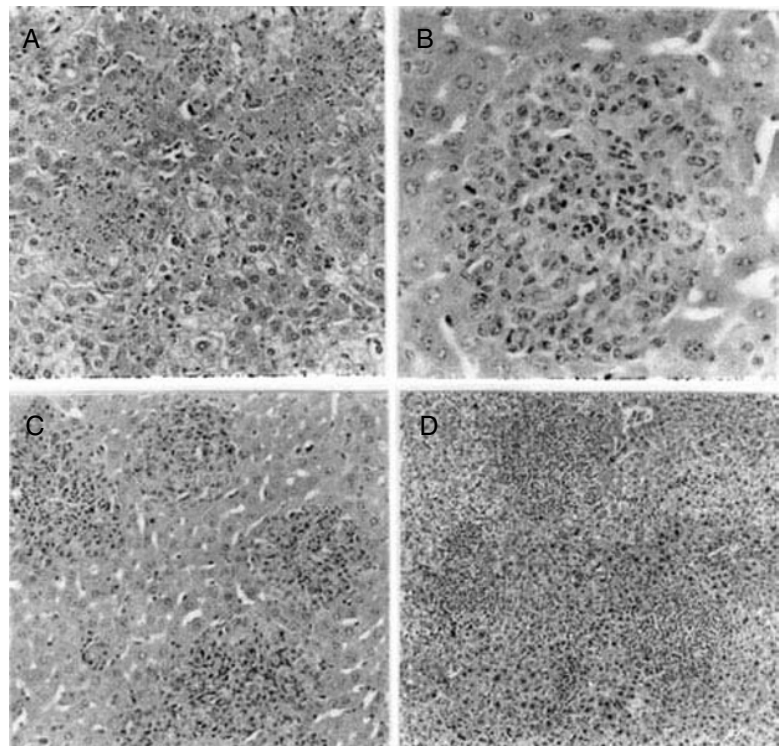


Fig. 8. Characteristic histopathological changes in liver caused by *Y. pestis* KIM10 on postinfection day 3. Hematoxylin and eosin stain was used. (A) Control mouse infected with pCD<sup>+</sup> yersiniae shows multiple necrotic focal lesions without inflammatory cell response (magnification,  $\times 140$ ); (B) Control mouse infected with pCD<sup>-</sup> yersiniae exhibits granuloma formation (magnification,  $\times 280$ ); (C) Mouse actively immunized with PAV and infected with pCD<sup>+</sup> yersiniae shows protective granulomatous lesions (magnification,  $\times 140$ ); and (D) Mouse passively immunized with polyclonal rabbit anti-PAV and infected with pCD<sup>+</sup> yersiniae shows pre-granulomatous lesions prompting infiltration of inflammatory (mononuclear) cells (magnification,  $\times 70$ ). From Nakajima et al. (1995).

sion. This type of disease, termed “septicemic plague,” is noted for a higher mortality rate (~90%) than the more typical bubonic form (~50%). Marked bacteremia also is characteristic of the final phase of bubonic plague. This symptom is ominous and generally reflects terminal colonization of liver and spleen. Another unfortunate and almost uniformly lethal variation of the human disease is pneumonic plague characterized by fever, cough and chest pain. The appearance of pneumonic plague during an epidemic is especially portentous because this variation of the disease permits direct transfer without reliance on the flea vector.

It sometimes has been suggested that the plague bacillus is now attenuated and no longer the scourge responsible for the Black Death. This notion was aptly challenged by Thomas Butler (Butler, 1983) who, as an isolated physician during the conflict in Vietnam, stated “... we were humbled by the swift progression of this disease that carried healthy individuals to death within three days.” One citation in Butler’s treatise noted that pneumonic plague in humans remains so severe that individuals may become ill and die on the same day they were infected. Antibiotics, however, can cure even this form of the disease if therapy is initiated promptly after exposure (Butler, 1983).

### Immunity

Both humoral and cellular mechanisms of immunity can provide protection against plague. Indeed, the disease is a race between the ability of the bacteria to cause death and the capacity of the host to mount a successful immune response. This contest is seldom won by the infected rodents (and often lost by humans), thus an appreciation of protective antigens is essential in understanding the nature of tissue invasion versus bulk vegetative growth in the viscera.

### Cell-Mediated Immunity

A live vaccine consisting of pCD<sup>+</sup>, Pgm<sup>-</sup> cells of *Y. pestis* EV76 provides effective immunity against plague. Carriage of pCD, however, is not necessary for protection by live cells, indicating that antigens other than those encoded by this plasmid are immunogenic (Burrows, 1963). Simonet et al. (1985a) showed that live pYV<sup>-</sup> cells of *Y. pseudotuberculosis* could also immunize against plague, thereby eliminating not only functions of pCD/pYV but also pPCP<sup>-</sup> and pMT-encoded activities as sole protective antigens. Though these results may reflect, in part, a contribution by circulating antibody, they also are in accord with the findings of Wong and Elberg (1977), who first demonstrated that fractions

of *Y. pestis* could initiate a classical T cell-modulated state of cellular immunity. It is now accepted that cell-mediated processes can provide significant protection against plague. Indeed, the ability to form protective granulomas, the hallmark of delayed-type sensitivity, assures the defeat of pathogenic yersiniae. However, preparations capable of promptly eliciting an effective cell-mediated response consist of whole bacteria or particulate matter and thus are apt to promote untoward inflammatory reactions. Consequently, investigators concerned with developing modern vaccines have concentrated on atoxic soluble subunit antigens possessing the ability to raise protective circulating antibodies.

### Humoral Immunity

A partially purified preparation of Caf1 generated protective antibodies against *Y. pestis* in mice (Baker et al., 1952); this and other reports in the early literature defining the ability of Caf1 to immunize against plague were reviewed by Burrows (1963). As already noted, Caf1 is not essential for lethality in mice (Burrows, 1957), monkeys (Davis et al., 1996) and probably humans (Winter et al., 1960). It is, therefore, not surprising that the degree of protection obtained upon immunization with Caf1 is modest (Andrews et al., 1996; Burrows, 1963; Meyer et al., 1974; Welkos et al., 1995), at least in comparison to that provided by LcrV (described below), which is known to be essential for expression of virulence. The ineffectiveness of Caf1 as a vaccine is heightened by the observation, first made by Burrows (1957), that Caf1<sup>-</sup> mutants constitute the terminal population of yersiniae recovered from mice succumbing to challenge with Caf1<sup>+</sup> *Y. pestis* after immunization with Caf1. Mutation to Caf1<sup>-</sup> was reported to occur at high frequency both in vitro and in vivo (Burrows and Bacon, 1958; Drozdov et al., 1995; Pirt et al., 1961), thus emphasizing its limitations as a soluble vaccine and determinative target for rapid identification.

The LcrV is superior to Caf1 determinant as a protective antigen as judged by its greater protective index and utter necessity as an effector of virulence (thereby eliminating the possibility of overgrowth by LcrV<sup>-</sup> mutants in vivo as occurs with Caf1<sup>-</sup> mutants). Results of initial studies showed that mice immunized with Lcr<sup>+</sup> strains generally exhibited greater immunity to subsequent challenge with wild-type yersiniae than occurred after immunization with Lcr<sup>-</sup> isolates (Burrows and Bacon, 1958). This observation was extended by Lawton et al. (1963) who showed that rabbit antiserum raised against a partially purified preparation of LcrV provided passive protection against plague in mice.

Attempts to prepare homogenous native LcrV by traditional methods of biochemical fractionation were unsuccessful due to the penchant of this protein to degrade spontaneously (Brubaker et al., 1987). Nevertheless, rabbit antisera prepared against the resulting mixture of highly purified peptides also provided passive immunity (Une and Brubaker, 1984a), allaying criticism that the active component present in the preparation of Lawton et al. (1963) was actually one or more contaminating antigens. More convincing evidence that anti-LcrV is protective was provided by Motin et al. (1994) who engineered a construct encoding a staphylococcal Protein A-LcrV fusion protein termed "PAV." After expression in *E. coli* and purification by affinity chromatography, homogenous PAV was used to immunize rabbits; the resulting antiserum provided significant passive protection against plague. Formal proof that immunity was, indeed, caused by anti-LcrV was provided by demonstrating that absorption of anti-PAV with excess native LcrV (or engineered truncated derivatives) eliminated the serum's ability to passively protect against disease. These experiments showed that at least one major protective epitope resided between amino acids 168 and 275. Protein A-LcrV fusion protein (PAV) was found to actively immunize against an intravenous challenge of  $-10^4$  pCD<sup>+</sup> cells of *Y. pestis* (Nakajima et al., 1995), whereas a homogenous preparation of hexahistidine-LcrV fusion protein (V<sub>h</sub>) purified by nickel affinity chromatography provided similar protection against  $-10^7$  bacteria (Motin et al., 1996). These observations have been amply verified and extended to provide the basis for an effective subunit plague vaccine consisting of LcrV alone (Anderson et al., 1996; Carr et al., 1999; Hill et al., 1997; Leary et al., 1995; Pullen et al., 1998) or in combination with CafI (Heath et al., 1997; Spiers et al., 1999; Williamson et al., 1999). In related studies, the LcrV homolog of *Pseudomonas aeruginosa* (PcrV) also was found to serve as a protective antigen (Sawa et al., 1999).

As noted above and under Physiology, loss of *lcrV* is pleiotropic in that these mutants fail to express or secrete effector Yops. Anti-LcrV could therefore provide immunity by mechanically blocking the type III translocation apparatus as reported for anti-PcrV in *Pseudomonas* (Sawa et al., 1999). In this case, other surface components of the secretion machine also might be expected to provide protection. Although the expression of Yops by *Y. pestis* in vitro is cryptic (Chalvignac et al., 1988; Kuttyrev et al., 1999; Mehig and Brubaker, 1993; Mehig et al., 1989; Sample and Brubaker, 1987a; Sample et al., 1987b; Straley and Brubaker, 1981; Straley and Brubaker, 1982), these proteins are clearly pro-

duced in vivo as first demonstrated by the presence of corresponding antibodies in convalescent human sera (Mazza et al., 1985). Anti-Yops antibodies also were observed upon study of mice surviving infection by aerosol following antibiotic therapy (Benner et al., 1999). Nevertheless, active immunization with the virulence effectors YopE and YopH or components of the secretion machine (YopK or LcrE) failed to provide significant protection (Andrews et al., 1999; Leary et al., 1999); YopD immunized against modest challenge with  $-2 \times 10^3$  yersiniae (Andrews et al., 1999). It is not surprising that antibodies directed against effector Yops fail to provide significant protection because the type III secretion apparatus effectively compartmentalizes these proteins from the immune system. However, it is not yet resolved why components of the secretion machine, itself, other than LcrV, fail to raise effective protective antibodies. One prospect is that, of the constituent proteins so far examined, only LcrV is accessible to antibodies. Another possibility is that the major physiological role of cytotoxic Yops is to promote focal tissue necrosis as shown in Fig. 7 and these non-vascularized lesions are inaccessible to circulating antibodies. Alternatively, LcrV might mediate a second unique function elsewhere required for virulence and neutralizable by circulating antibody. An observation favoring the latter alternative is that anti-LcrV restores the ability of the host to mount a generic inflammatory response (Fig. 6D) characterized by normal expression of IFN- $\gamma$  and TNF- $\alpha$  (Nakajima and Brubaker, 1993).

Pettersson et al. (1999) presented data indicating that LcrV appears on the bacterial surface before contact with the host target cell and that its interaction with specific antibody blocks subsequent translocation of effector Yops. Unfortunately, these results could not be verified by Fields et al. (1999b) who also observed the presence of LcrV in punctate zones on the bacterial surface but found that an otherwise protective anti-LcrV serum failed to inhibit pCD-mediated translocation of YopE by docked yersiniae. As noted below, the ability to express IFN- $\gamma$  and TNF- $\alpha$  as a function of stress was prevented by recombinant LcrV (Nakajima et al., 1995). Downregulation of these important proinflammatory cytokines by recombinant LcrV also was detected in cell culture (Schmidt et al., 1999). Evidence is summarized below indicating that the ability of anti-LcrV serum to provide immunity reflects its ability to restore the generic inflammatory response. Additional antigens capable of raising protective antibodies against plague undoubtedly exist. For example, anti-Pla serum was found to provide passive immunity against subcutaneous infection presumably by

preventing plasmin-mediated metastasis of invading yersiniae (Kutyrev and Brubaker, 1999).

### Virulence Factors Required for Dissemination in Tissues

Studies of the disease process outlined immediately above demonstrated that plague bacilli first access favored sites of multiplication within the viscera from dermis and then commence a massive and prolonged process of vegetative growth. This second phase seriously jeopardizes host homeostasis by causing eventual loss of organ function accompanied by spillage of the organisms into the vascular system. Determinants of virulence that facilitate the first invasive phase of plague are not always involved in the second vegetative phase and vice versa. Furthermore, cells of *Y. pestis*, as well as the enteropathogenic yersiniae, can cause acute disease following intravenous injection but only plague bacilli possess the ability to efficiently access internal organs from the peripheral sites of infection. This journey is tumultuous as judged by the larger inocula of enteropathogenic yersiniae required to cause death by subcutaneous or intradermal administration (Table 3). Obviously plague bacilli easily foil nonspecific processes of host defense that waylay enteropathogenic yersiniae en route. Indeed, it is the virulence factors of the first invasive phase that largely account for the acute nature of bubonic plague.

Yersiniae pathogenic to humans, like most Gram-negative bacterial pathogens, are innately indifferent to lysozyme and  $\beta$ -lysin. Cells of *Y. pseudotuberculosis* grown at 37°C are resistant to complement-mediated killing, whereas those cultivated at 26°C are sensitive; this property is constitutive in *Y. pestis* (Une and Brubaker, 1984b). Similarly, smooth LPS is elaborated by cells of *Y. pseudotuberculosis* grown at 26°C that possesses O-group structures containing immunodominant 3,6-dideoxyhexoses (Samuelsson et al., 1974). These O-groups are not formed during growth at host temperature and an analogous rough LPS is constitutively expressed by plague bacilli (Federova and Devdariani, 1998; Minka and Bruneteau, 1998; Porat et al., 1995). Liposomes composed of rough LPS from these two species were resistant to complement-mediated lysis, whereas those formed from the smooth LPS of *Y. pseudotuberculosis* grown at 26°C were sensitive (Porat et al., 1995). This result was not dependent upon carriage of pCD/pYV, although only pYV<sup>+</sup> cells of *Y. enterocolitica* grown at 37°C (and liposomes prepared with LPS from these bacteria) were resistant to complement (Porat et al., 1995; Une and Brubaker, 1984a). The pYV-encoded YadA adhesin of the enteropathogenic

yersiniae was reported to prevent complement-mediated killing (Finlay and Falkow, 1997) but probably does not account for serum-resistance of liposomes. In any event, neither YadA nor invasins are expressed by *Y. pestis*, leaving no other alternative at present but to assign serum-resistance of this species to rough LPS structure.

Considerable controversy surrounds the ability of yersiniae pathogenic to humans to avoid uptake by professional phagocytes or to survive intracellularly following this event. Reports of studies comparing these abilities in *Y. pestis* and the enteropathogenic yersiniae under identical conditions have not yet appeared; thus, results obtained with one species may not apply to another. Once ingested by professional phagocytes, cells of *Y. enterocolitica* cured of pYV fare poorly, emphasizing the importance of pYV-mediated functions in promoting resistance to intracellular killing (Hartland et al., 1996; Hartland et al., 1994; Lian and Pai, 1985; Ruckdeschel et al., 1996). However, pYV<sup>-</sup> cells of *Y. pseudotuberculosis* grew as rapidly within primary macrophages of spleen and kidney as did those of the pYV<sup>+</sup> parent (Richardson and Harkness, 1970). In contrast, Simonet et al. (1985b) demonstrated that carriage of pYV by *Y. pseudotuberculosis* neither facilitated resistance to ingestion by mouse resident macrophages in vitro nor inhibited the oxidative burst of macrophages. Extension of this approach to the early stages of infection in mice showed that both pYV<sup>+</sup> and pYV<sup>-</sup> organisms were eliminated at equal rates from the liver and the peritoneal cavity. This finding suggested that pYV-mediated factors were either not effective in preventing intracellular killing or not expressed until after a significant latent period. Phagocytosis of pYV<sup>+</sup> cells of *Y. pseudotuberculosis* by a monocyte-macrophage cell line (J774A.1) resulted in normal degranulation and formation of phagolysosomes within which the bacteria were killed (Mills et al., 1997). Salmonellae, but not *E. coli*, not only survived this ordeal but also increased five-fold in number during the process. The Caf1 antigen of *Y. pestis* confers modest resistance to uptake by professional phagocytes, especially neutrophils and monocytes, during the initial invasion of tissues. However, this capsular antigen probably is ineffective against fixed macrophages lining the capillary network of liver and spleen as judged by the ability of both Caf1<sup>+</sup> and Caf1<sup>-</sup> strains to initiate growth in these organs following intravenous injection. Both pCD<sup>+</sup> and pCD<sup>-</sup> plague bacilli survived equally well within free macrophages, at least at low multiplicities (Cavanaugh and Randall, 1959; Charnetzky and Shuford, 1985; Goguen et al., 1986; Janssen and Surgalla, 1969; Straley and Harmon, 1984). These observations suggest that pCD/pYV-mediated



functions are insufficient for survival of *Y. pseudotuberculosis* in free phagocytes and superfluous for such survival of *Y. pestis*. Further work will be necessary to verify the impression that carriage of pCD/pYV is unnecessary for rapid growth of *Y. pestis* and *Y. pseudotuberculosis* within fixed macrophages.

Even though effector Yops do not mediate intracellular survival of *Y. pseudotuberculosis* or *Y. pestis*, their translocation can render professional phagocytes incapable of ingestion (Anderson et al., 1999; Fallman et al., 1995; Persson et al., 1997; Persson et al., 1995; Rosqvist et al., 1990) or cause them to undergo apoptosis (Mills et al., 1997; Monack et al., 1998; Monack et al., 1997; Ruckdeschel et al., 1997). Nevertheless, it is only at higher multiplicities that pCD-mediated cytotoxic Yops assure prompt destruction of professional and nonprofessional phagocytes (Charnetzky and Shuford, 1985; Goguen et al., 1986). Considered in this sense, these effectors are correctly viewed as leukocidins. However, plague bacilli may not achieve multiplicities sufficiently high to destroy professional phagocytes during migration from peripheral sites of infection to visceral organs except during invasion of regional lymph nodes. This confrontation prompts cytolysis and tissue destruction leading to formation of buboes. The organisms are otherwise viewed with seeming indifference by the host and, upon arrival at their destination in visceral organs, proceed to initiate bulk vegetative growth within focal necrotic lesions. Further study will be necessary to define the basis of this prompt and safe passage. The absence of YadA, Inv and Ail minimizes bacterial interaction with both professional and nonprofessional phagocytes. This deficiency thus hastens the journey while reducing expression of proinflammatory cytokines/chemotaxins induced by contact with host cells.

Two additional determinants are required for successful invasion of the viscera. As shown in Table 3, the first is pPCP-encoded Pla and the second is the ability to express the siderophore yersiniabactin encoded by genes resident to the high-pathogenicity island mapping within the *pgm* locus (described under Physiology). The simplest explanation for the requirement of Pla is that this outer-membrane tissue invasin promotes adherence to the mammalian extracellular matrix, thereby facilitating bacterial metastasis (Lähteenmäki et al., 1998). The finding that mutation to Pgm<sup>-</sup> (and thus loss of ability to synthesize yersiniabactin) also is necessary for invasion probably indicates that iron is in especially short supply in peripheral tissue and cannot be adequately assimilated without use of this high-affinity mechanism of transport. Further study will be required to determine whether KatY,

indeed, functions as a virulence factor as might be anticipated by its abundant temperature-dependent production and, if so, whether this activity facilitates survival within professional phagocytes or serves some alternative function.

### Virulence Factors Required for Vegetative Growth in Visceral Organs

The comparisons between the pathology of murine and human plague outlined above provide three insights. First, the diseases in both hosts are similar except that the mouse succumbs before exhibiting comparable lymphadenopathy. Second, death occurs only after the bacteria have undergone numerous doublings in visceral organs. Third, the ability of all yersiniae (pathogenic to humans) to sustain this assault is dependent upon carriage of pCD/pYV as shown by the observation that mutants lacking these plasmids (or lacking certain genes encoded thereon) are rapidly cleared from liver and spleen (Fig. 7). This vegetative phase of the disease involves the destruction of tissue comprising these organs and subsequent metabolism of nutrients within the released cytoplasm. Cell disintegration is probably accomplished exclusively by the pCD-encoded type III protein secretion system described under Physiology that translocates cytotoxic Yops from the cytoplasm of docked yersiniae into target nonprofessional phagocytes. This process functions without exposing the virulence effectors to the extracellular environment and is tightly regulated by temperature, metallic cations, and ability to form an intact structural unit required for translocation. The remarkable feature of this mechanism is that the host permits it to occur. Tissue damage of this extent invariably causes inflammation accompanied by release of chemotaxins and proinflammatory cytokines that lead to prompt infiltration of mononuclear cells capable of containing the invading bacteria within protective granulomas. Formation of granulomas is the last nonspecific resort of the host in defending against bacterial invasion. However, containment within granulomatous lesions is extraordinarily effective in that physiological changes within the enclosed environment are extreme and highly bactericidal as might be expected of a process used to control infection caused by many facultative intracellular parasites. In short, cells of *Y. pestis* lacking pCD promptly undergo containment and then destruction within granulomas during residence in visceral organs. However, carriage of pCD both facilitates tissue necrosis and prevents infiltration of inflammatory cells, thereby assuring unrestricted growth (Nakajima et al., 1995; Une et al., 1986).

The LcrV protein inhibits normal production of IFN- $\gamma$  and TNF- $\alpha$  (Fig. 6) as judged by the

ability of anti-LcrV to restore the generic inflammatory response (Nakajima and Brubaker, 1993). Furthermore, an engineered staphylococcal Protein A-LcrV fusion protein termed PAV, expressed in *E. coli* and purified to homogeneity by IgG affinity chromatography (Motin et al., 1994), mimicked the ability of whole pCD<sup>+</sup> yersiniae to downregulate IFN- $\gamma$  and TNF- $\alpha$  (Nakajima et al., 1995). These results, shown in Fig. 9, strongly indicate that LcrV per se accounts for downregulation. Schmidt et al. (1999) showed that a LcrV-polyhistidine fusion protein engineered from *Y. enterocolitica* downregulated LPS-induced TNF- $\alpha$  in mouse spleen and liver. This inhibition occurred at the level of mRNA synthesis, was not associated with upregulation of interleukin-10, could be blocked by specific antibody in tissue culture, and was dependent upon the presence of both macrophages and activated T cells. Considered together, these results suggest that LcrV per se prevents the host from mounting an effective inflammatory response against plague bacilli. However, as noted above, others have suggested that translocated Yops, especially YopH and YopJ, also promote this effect (Mills et al., 1997; Monack et al., 1998; Palmer et al., 1998; Schesser et al., 1998; Yao et al., 1999). Though suppression of macrophage-induced TNF- $\alpha$  by YopJ is tenable, its mode of action would not be expected to downregulate IFN- $\gamma$ , which is primarily expressed by T<sub>H</sub>1 cells. Indeed, YopJ- mutants retain the ability to suppress IFN- $\gamma$  and TNF- $\alpha$  in a manner identical to that shown in Fig. 6B for pCD<sup>+</sup> yersiniae (R. Brubaker, unpublished observations). The YopH protein can modulate a wide variety of T and B cell-mediated responses by downregulating the co-stimulatory molecule B7.2 (Yao et al., 1999) and thus undoubtedly helps maintain the anti-

inflammatory umbrella typical of plague. Note, however, that docking of yersiniae to host cells is necessary for initiation of anti-inflammatory activities provided by YopH and YopJ; thus their effects will necessarily remain localized as opposed to that of LcrV which is active in extracellular soluble form (Motin et al., 1997; Nakajima et al., 1995; Nedialkov et al., 1997).

Whereas YopJ and YopH undoubtedly contribute to downregulation of IFN- $\gamma$  and TNF- $\alpha$ , these and other Yop effectors also account for the pCD-mediated focal lesions of liver and spleen that are characteristic of infection by *Y. pestis* and *Y. pseudotuberculosis*. Tissue damage of this extent would not otherwise be tolerated by the host and would be promptly controlled by normal nonspecific processes assuring infiltration of inflammatory cells and subsequent formation of granulomatous lesions. However, owing to the ability of yersiniae to downregulate proinflammatory cytokines, the host is unable to initiate the steps necessary to provide effective containment. This bleak outlook is entirely in accord with the observation of Welkos et al. (1998) that LcrV blocks chemotaxis of neutrophils. Consequently, yersiniae progressively enlarge the focal lesions within which they are contained and eventually overwhelm organ function causing death. Subtle but probably significant differences exist between the focal necrotic lesions caused by plague bacilli within visceral organs and those formed by *Y. pseudotuberculosis* (Nakajima et al., 1995). The latter resemble abscesses in that they are surrounded by neutrophils, which nevertheless are ineffective in containing the infection. The explanation for this difference is unknown. One possibility is that the anti-inflammatory umbrella provided by LcrV for *Y. pseudotuberculosis* might be leaky. That is, either YadA/invasin-host cell interaction, extra-

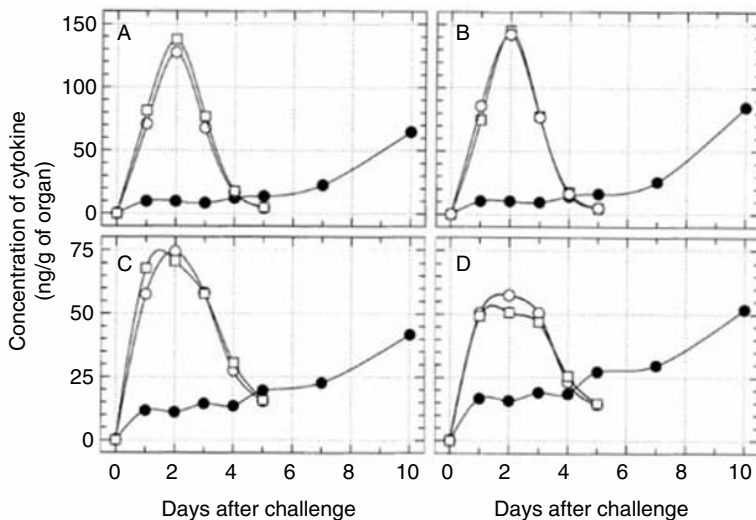


Fig. 9. Levels of IFN- $\gamma$  in spleen (A) and liver (B) or of TNF- $\alpha$  in spleen (C) or liver (D) of outbred (Swiss-Webster) mice following induction of cytokine synthesis on day 0 by intravenous injection of  $10^6$  avirulent pCD<sup>-</sup> cells of *Y. pestis* KIM. Mice received daily injections of 0.1 ml of 0.033 M potassium phosphate buffer, pH 7.0 (□), or 100  $\mu$ g of either Protein A (○) or Protein A<sup>-</sup> LcrV (PAV) fusion protein (●) in 0.1 ml of the same buffer on postinfection days 0 through 5. Values represent averages determined for four mice. Data from Nakajima et al. (1995).

cellular accumulation of undelivered Yops (expressed in the  $\text{Ca}^{2+}$ -deficient environment of released host cell cytoplasm), or both could provide sufficient stimulation to enable the host to overcome the anti-inflammatory effects of LcrV, YopJ and YopH. These events would constitute the basis for environmental pressure favoring the selection of mutants lacking YadA, Inv and Ail and furnish an additional advantage for expression of Pla.

## Applications

Students of molecular pathogenesis are nowadays forewarned that new infectious diseases will almost surely arise and old afflictions such as the pestilence will re-emerge. These specters are very real, as is the threat that the old epidemic diseases of humans will appear again in even more sinister forms (agents of biological warfare and terrorism). To prevent or at least minimize these bleak scenarios, many governments presently sponsor laudable programs to design effective methods to identify highly pathogenic species and develop vaccines to prevent the diseases that they cause. The silver lining to the present focus on these onerous aspects of medical microbiology is that investigators are becoming increasingly aware that the gene pools of highly pathogenic species encode astonishing biological activities that possess the potential to reverse the pathology of noninfectious processes of disease. The LcrV protein is an excellent example of this phenomenon.

Perry et al. (1998) noted that LcrV is the only pCD-encoded virulence factor capable of mediating biological effects in soluble form from host fluids. These responses all reflect the penchant of this protein to downregulate proinflammatory cytokines (Nakajima et al., 1995; Schmidt et al., 1999) and thereby minimize inflammation. This effect, of course, is not always beneficial in that injected PAV significantly prolonged the retention of LcrV<sup>-</sup> mutants in vivo and greatly enhanced the ability of unrelated bacteria (especially *Listeria monocytogenes*) to cause serious disease (Nakajima et al., 1995). Prolonged administration of PAV to normal mice did not, however, result in overgrowth by the normal flora. These determinations were undertaken to compare the retention of skin allografts in mice so as to distinguish pure anti-inflammatory activity resulting from downregulation of proinflammatory cytokines from inhibition of specific immunity (Motin et al., 1997). No significant difference was noted between treated and control mice with regard to time of total allograft retention, but the period before onset of visible inflammation was doubled from 6 to 12 days

( $p < 0.001$ ). This finding illustrates that LcrV is indeed anti-inflammatory but does not interfere with the onset of specific cell-mediated immunity per se (other than inhibiting cytokine-mediated processes common to both mechanisms). It is established that endotoxic shock is a consequence of tissue damage resulting from excessive stimulation by proinflammatory cytokines. An LPS-free polyhistidine-tagged LcrV fusion protein (Motin et al., 1996) promoted an immediate resistance to LPS that rapidly dissipated and then reappeared after 48 hours (Nedialkov et al., 1997). Downregulation of IFN- $\gamma$  and TNF- $\alpha$  occurred during both phases, as did eventual upregulation of anti-inflammatory cytokines, especially IL-10 (generally known to mediate LPS tolerance). The early phase of resistance could not represent LPS tolerance (which requires 48 hours for full expression); thus, immediate downregulation of IFN- $\gamma$  and TNF- $\alpha$  appeared to reflect some novel LcrV-mediated ability to promptly upregulate anti-inflammatory cytokines, thereby preventing inflammation. The interpretation that LcrV functions by upregulating IL-10 is probably fallacious as judged by the finding of Schmidt et al. (1999) that anti-IL-10 did not influence the ability of LcrV to immediately downregulate IFN- $\gamma$  and TNF- $\alpha$  (although upregulation of anti-inflammatory cytokines may still account for the delayed phase of resistance).

Any mechanism capable of inhibiting the expression of proinflammatory cytokines has obvious potential applications in the treatment of inflammatory diseases such as arthritis, rheumatism and septic shock. Although the ability of PAV to postpone (but not prevent) inflammation associated with allograft rejection was impressive, of equal interest was the observation that the grafts "set" immediately without exhibiting erythema, edema or other signs of inflammation, which suggests an application in the healing of wounds. Further study may show that Pla has similar applications in the treatment of noninfectious processes. The possibility that this protein can serve as an alternative to other plasminogen activators in the treatment of cardiovascular disease is obvious. However, the ability of Pla to generate plasmin at the level of the basement membrane and thereby promote bacterial metastasis by dissociating host cells is unique (Lähteenmäki et al., 1998). It will be interesting to see if this activity can similarly dissociate solid tumor cell masses, thereby facilitating normal chemotherapeutic regimens. It is entirely likely that these and other appropriately cloned and purified virulence factors of yersiniae, as well as analogues from other pathogenic species, will see widespread use as therapeutic agents in the near future.

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## *Erwinia* and Related Genera

CLARENCE I. KADO

### Introduction

Members of the genus *Erwinia* are primarily plant-pathogenic and plant-associated bacteria. As modern approaches have focused on the direct analysis of genes and their gene products, commonly associated phenotypes such as the type of disease that they cause and the relatedness of rDNA have prompted splitting of certain *Erwinia* members into the genus *Enterobacter*, *Pectobacterium*, *Pantoea* or *Brenneria*. For example, the genus *Pectobacterium*, which was suggested previously (Brenner et al., 1973), has been resurrected to accommodate those species that profusely produce pectinolytic enzymes in plant pathogenesis and that have related rDNA (Hauben et al., 1998). Because of the large difference in the percent G+C and the rDNA sequence, the genus *Pantoea* was added. As the sequences of entire genomes of this group of organisms become available, regrouping of the members of these genera will likely take place. Pairwise 16S rDNA sequence comparisons also showed *E. alni*, *E. nigrifluens*, *E. paradisiaca*, *E. quercina*, *E. rubrifaciens* and *E. salicis* grouped into the new genus *Brenneria* (Hauben et al., 1998).

### *Erwinia*

The genus *Erwinia* is classified in the *Enterobacteriaceae*. Most members of this genus characteristically cause diseases of plants. Recent 16S rDNA sequence comparisons have been proposed to delineate species originally classified in the genus *Erwinia* into the genus *Pantoea*, *Enterobacter*, *Pectobacterium*, or *Brenneria*. Certain species with the distinct glucose metabolism of oxidizing D-glucose into various forms of gluconate have been removed from the genus *Erwinia* and moved into the genus *Pantoea*. All of the genera possess the main phenotypic characteristics of the *Enterobacteriaceae*. They produce acid from sugars, are Gram-negative rods bearing peritrichous flagella, and can ferment

substrates anaerobically. The phylogenetic position of the genus *Erwinia*, along with other members of the *Enterobacteriaceae* associated with plants, have been explored using 16S rDNA nucleotide sequence comparisons (Kwon et al., 1997; Hauben et al., 1998). Four phylogenetic groups representing a branch of the lineage tree have been proposed. One group comprises *E. amylovora*, *E. rhapontici*, *E. persicina*, *E. psidii*, *E. pyrifoliae*, *E. mallotivora* and *E. tracheiphila*. The second group consists of *E. carotovora* subspecies, *E. chrysanthemi*, and *E. cyripedii*. The latter group was proposed to be moved to the genus *Pectobacterium* primarily because it produces pectolytic enzymes for pathogenesis (for history see Brenner et al., 1973). The third group comprises *E. alni*, *E. nigrifluens*, *E. rubrifaciens*, *E. paradisiaca*, *E. quercina*, and *E. salicis*. It was suggested this third group be moved into the proposed genus *Brenneria* (Hauben et al., 1998). The fourth group comprises members that display unusual oxidative metabolism of D-glucose and produce yellow to mauve colonies. They were reclassified into the genus *Pantoea* (Gavini et al., 1989; Kageyama et al., 1992; Mergaert et al., 1993). The generic positioning of *Enterobacter*, *Pectobacterium*, *Pantoea*, and *Brenneria* was essential to avoid further confusion in sorting species previously classified within the genus *Erwinia*. A more solid positioning will be forthcoming when the entire genome of each of these genera is completed and comparisons of each of the genomic sequences are made.

### Habitat

*Erwinia*, *Enterobacter*, *Pectobacterium*, *Pantoea* and *Brenneria* species cause four basic types of plant diseases: 1) rapid necrosis; 2) progressive tissue maceration called “soft-rot;” 3) occlusion of vessel elements called “vascular wilt,” and 4) hypertrophy leading to gall or tumor formation. A list of species with these characteristic phenotypes is shown in [Table 1](#).

Table 1. *Erwinia* and subspecific genera and their disease-causing types.

Species	Disease name	Type of infection	Natural host plant
Necrogenic group emended from <i>Erwinia</i> to <i>Brenneria</i> (Hauben et al., 1998)			
<i>Brenneria alni</i> Wilson et al., 1957	Canker	Necrogenic	Alder
<i>B. nigrifluens</i>	Shallow canker	Necrogenic	Members of the Juglandaceae
<i>B. paradisiaca</i>	Brown-black root rot	Necrogenic	Banana
<i>B. quercina</i> Hildebrand and Schroth, 1967	Drippy nut and blight	Maceration	Coast live oak ( <i>Quercus agrifolia</i> )
<i>B. rubrifaciens</i> Wilson et al., 1967	Deep bark canker	Necrogenic	Members of the Juglandaceae
<i>B. salicis</i> (Day 1924) Chester 1939	Bacterial wilt, water mark	Vascular wilt	<i>Salix</i> species
Status quo <i>Erwinia</i> group			
<i>E. amylovora</i> (Burrill, 1882) Winslow et al., 1920	Fire blight	Necrogenic	Members of the Rosaceae
<i>E. billingia</i> Mergaert et al., 1999	Associated with cankers	Secondary invader	Members of the Rosaceae
<i>E. cypripedii</i> (Hori) Bergey et al., 1923	Brown rot	Maceration	<i>Cypripedium</i> and other orchids
<i>E. mallotivora</i> Goto, 1976	Black leaf spot	Necrogenic	<i>Mallotus japonicus</i> tree
<i>E. persicinus</i>	Red-brown fruit rot	Necrogenic	Cucumber, tomato, banana
<i>E. psidii</i>	Fruit rot	Necrogenic	Guava
<i>E. pyrifoliae</i> Kim et al., 1999	Leaf and stem blight	Necrogenic	Pear, <i>Pyrus pyrifolia</i> cv Shingo
<i>E. rhapontici</i> (Millard) Burkholder 1948	Crown rot	Maceration	Rhubarb
<i>E. tracheiphila</i> (Smith, 1895) Bergey et al., 1923	Cucumber wilt	Vascular wilt	<i>Cucumis</i> species
<i>P. carotovora</i> subsp. <i>Carotovora</i> (Jones) Bergey et al., 1923	Storage soft rot	Maceration	Harvested corms, tubers, roots, bulbs
<i>P. carotovora</i> subsp. <i>Chrysanthemi</i> Burkholder et al., 1953	Stem blight, wilt	Vascular wilt	Corn, carnation, chrysanthemum, tropic plants, e.g., <i>Philodendron</i> , <i>Dieffenbachia</i>
<i>P. carotovora</i> subsp. <i>atroseptica</i> (van Hall 1902) Dye 1969	Blackleg	Vascular wilt	Potato roots, lower stems, tubers
<i>P. carotovora</i> subsp. <i>betavascularum</i>	Soft rot	Maceration	Beet, <i>Beta vulgaris</i>
<i>P. carotovora</i> subsp. <i>odoriferum</i>	Soft rot	Maceration	Endive and chicory
<i>P. carotovora</i> subsp. <i>wasabiae</i>	Soft rot of excised petioles	Maceration	Japanese green horse radish
Erwinia species emended to Enterobacter species			
Enterobacter cloacae	Internal yellowing, brown-black discoloration	Diffuse necrogenic	Papaya ( <i>Carica papaya</i> L.) Onion ( <i>Allium cepa</i> L.)
En. nimipressuralis	Wet wood	Necrogenic	Elm
En. cancerogenus	Canker	Necrogenic	Poplar
En. dissolvens	Rot	Slow maceration	Corn

Soft-rotting group proposed emendation from *Erwinia* to *Pectobacterium* (Hauben et al., 1998).

## Isolation

*Erwinia* species and species of its subspecific genera can be isolated from diseased tissues most expeditiously from fresh samples. Most *Erwiniae* are not fastidious with regard to the medium used, but it has been a general practice to employ relatively rich media such as Luria-Bertani (LB) agar, which contains per liter 10g tryptone (Difco), 5g yeast extract, 5g sodium chloride, 15g agar; and medium 523, which contains per liter 10g sucrose, 8g tryptone, 4g yeast extract, 2g dipotassium phosphate, 0.3g magnesium sulfate, and 15g agar. Supplements, such as 1% sorbitol, are added to LB agar medium for *E. amylovora* and related species. Although LB has been the most popular agar medium for isolation, nutrient agar (NA), which contains per liter 3g beef extract, 5g peptone, 8g sodium chloride and 15g agar, also has been used. Since NA was designed as general purpose medium for the isolation of mammalian bacterial pathogens, its use is not recommended for *Erwinia* and its subgeneric species that are associated with plants. For example, *Pantoea* species grow poorly on this medium. Some laboratories routinely have used NA supplemented with glycerol to compensate for poor growth of *Erwinia* species. This is poor practice. *Pantoea* species generally will grow more profusely when the medium is supplemented with nicotinic acid. For *Erwinia* species that produce excessive acid, medium YGC is used. Medium YGC agar contains per liter 20g glucose, 10g yeast extract, 20g ultrafine calcium carbonate and 15g agar. A clear zone surrounding each colony results from acid secretion, which liberates the carbonate as CO<sub>2</sub>. The usual temperature for culture of *Erwinia* and its generic members is 28°C. An incubator with anti-desiccation features such as a humidifier (e.g., Kendro HeraCell) is preferable. Agar plates are incubated in the inverted position.

## Identification

*Erwinia* and its subgeneric members usually are motile rods bearing peritrichous flagella and are able to ferment glucose leading to the formation of acid. Their fermentative pathway yields mixed acids and 2,3-butanediol. They are unable to utilize starch as a carbon source except for *E. rhapontici*, which produces a weakly diffusible pink pigment called ferrosamine (Feistner et al., 1983). *E. rubrifaciens* (*B. rubrifaciens*) produces a water soluble, diffusible red pigment called rubrifacine A (Fistner et al., 1984) on yeast-glucose calcium carbonate agar. Some strains of *Pectobacterium chrysanthemi* produce yellow colonies streaked with a green-blue pig-

ment. All are catalase positive and negative for exocellular cytochrome oxidase activity. Except for the *Erwinia* species that cause soft rot, the necrogenic and vascular wilting group reduce nitrate. On LB or 523 media, their colonies are generally mucoid and domed and can vary in color from white to cream.

Members of the genus *Pantoea* produce colonies that may appear either as yellow to taupe or mauve on LB agar. In contrast to the *Erwinia* group, *Pantoea* species oxidize glucose to gluconate. The genus *Pantoea* includes phytopathogens *P. stewartii*, *P. ananas*, *P. citrea* and *P. agglomerans* pv. *milletiae*, *P. agglomerans* pv. *gypsophilae*, and *P. agglomerans* pv. *betae*. *P. ananas* (*P. ananatis*) causes brown patches on internal portions of pineapple fruit [*Ananas comosus* (L.) Merr.] and honeydew melon (*Cucumis melo* L.). The disease in pineapple is called "marbling." *P. uredovora* first was described as an epiphyte on uredospores of *Ustilago* smut of maize and on the panicles of barley, buckwheat and rice. Based on recent 16S rDNA sequence data, *P. ananas* and *P. uredovora* are homologous and, therefore, the specific name *P. ananas* takes precedence with *P. uredovora* as a retired species name. *P. ananas*, changed to *P. ananatis* (Hauben et al., 1998) is a Gram-negative, rod-shaped, facultative anaerobe and usually forms yellow, semimucoid colonies on YGC agar. Some strains contain the ice nucleation gene *inaA*, somewhat similar to *inaW* and *inaZ* genes of *Pseudomonas syringae* and *P. fluorescens*. *Pantoea citrea* causes pink disease of pineapple (Cha et al., 1998). *P. citrea* is Gram-negative, rod-shaped, facultative anaerobe, and produces pili. It causes the hypersensitivity response in tobacco and, therefore, contains *hrp* genes. On nutrient agar and trypticase soy agar, the colonies are entire, smooth, glistening, translucent, but not mucoid. As the colonies age, they turn taupe with a slight depression in the center. *P. citrea* produces two quinoprotein glucose dehydrogenases encoded by *gdhA* and *gdhB* genes (Pujol and Kado, 1999). The glucose dehydrogenase encoded by *gdhB* efficiently converts glucose into gluconate, which, in turn, is oxidized to 2-ketogluconate. This latter substrate is further oxidized into 2,5-diketogluconate, a highly unstable compound that is chromogenic. *P. punctata* and *P. terrea* can oxidize glucose via the *gdhA* pathway. *P. citrea* harbors a cryptic plasmid with 5,229 base pairs. The plasmid, designated pUCD5000, is required for the full expression of the pink disease (Pujol and Kado, 1998).

*P. stewartii* subsp. *stewartii* causes Stewart's bacterial wilt and leaf blight disease of corn. This bacterium produces copious amounts of exopolysaccharides, the profuseness of which is believed to cause plugging of vessel elements in the host plant. Beginning symptoms are water-

soaked lesions, followed by vascular wilting on seedlings and leaf blight of mature plants. Some strains that cause leaf spots on foxtail millet (*Setaria italica*) and pearl millet (*Pennisetum americanum*) have been placed in *P. stewartii* subsp. *indologenes* (Mergaert et al., 1993). Multiple numbers of cryptic plasmids are co-residents in *P. stewartii* subsp. *stewartii*. The replication and mobilization loci in cryptic plasmids pSW100 and pSW200 from *P. stewartii* subsp. *stewartii* share sequence homologies with pUCD5000 from *P. citrea*. The habitats of members of the genus *Pantoea* are listed in Table 2. The physiological and biochemical characteristics of *Erwinia* and its related genera are shown in Table 3.

## Cultivation

*P. citrea* may be cultured on MGY medium (10g mannitol, 2g of sodium L-glutamate, 0.5g of monobasic potassium phosphate, 0.2g of sodium chloride, 0.2g of magnesium sulfate, 0.25g yeast extract per liter, adjusted to pH 7.0 with 3 N NaOH) at 30°C. The rusty red color produced by *P. citrea* may be generated in canned pineapple juice. The juice is clarified by centrifugation (12,000 × g, 10 min, 4°C) and adjusted to pH 6.0 with 3 N NaOH. The clarified juice is sterilized by filtration (0.4 μm-pore-size polycarbonate membrane filter; Phoretics Corporation, Livermore, California).

Table 2. Habitat and disease produced by members of the *Pantoea* group.

Species	Disease name	Disease type	Natural host
<i>P. agglomerans</i> pv. <i>agglomerans</i>	Stem darkening	Secondary	Celery
<i>P. agglomerans</i> pv. <i>betae</i>	Root gall	Tumorigenesis	Table beets <i>Beta vulgaris</i> L.
<i>P. agglomerans</i> pv. <i>gypsophilae</i>	Bacterial gall	Tumorigenesis by indole-3-acetic acid production	Baby's breath <i>Gypsophila paniculata</i> L.
<i>P. agglomerans</i> pv. <i>milliteae</i>	Bacterial gall	Tumorigenesis	Japanese wisteria <i>Wisteria floribunda</i>
<i>P. ananatis</i>	Marbling	Soft rot	Pineapple, sugarcane, honey dew melon
<i>P. citrea</i>	Pink disease	Translucent rot	Pineapple, mandarin orange
<i>P. dispersa</i>	Saprophyte	None	Edaphosphere resident, seeds
<i>P. punctata</i>	Brown spot	Delayed maceration	Mandarin orange
<i>P. stewartii</i> subsp. <i>indologenes</i>	Brown spot	Necrogenic	Graminae
<i>P. stewartii</i> subsp. <i>stewartii</i>	Stewart's wilt	Vascular wilt	Corn
<i>P. terrea</i>	Saprophyte	None	Soil inhabitant

Table 3. Comparative physiological and biochemical characteristics of *Pantoea* spp. versus *Erwinia* spp.

Characteristic	<i>Erwinia</i> spp.	<i>Pantoea</i> spp.	<i>Pectobacterium</i> spp.	<i>Brenneria</i> spp.	<i>Enterobacter</i> spp.
Gram-negative rods	+	+	+	+	+
Motile by peritrichous flagella	+	+/-	+	+	+
Facultative anaerobe	+	+	+	+	+
Colonies yellow or mauve	-	+	-	-	-
Percent genomic G+C content	49.8–54.1	55.1–60.6	50.5–56.1	50.1–56.1	52.0–54.0
Gluconate dehydrogenase	-	+	-	-	-
Cytochrome oxidase released	-	-	-	-	-
Catalase released	+	+	+	+	+
Indole production	-	+/-	-/+	-	-
Nitrate reduced to nitrite	+	+/-	+	-	+
H <sub>2</sub> S produced from cysteine	+/-	-	+	+	-
Urease produced	-	-	-	-/+	-
Gas produced from glucose	-	-	+	-	+
Ornithine decarboxylase	-	-	-	-	+
Lipase	-	+	-	-	-
Acid from α-methylglucoside	+	-	-	-/+	-
Gelatin liquefaction	-/+	-	+	-	+
Acid from sorbitol	+	-	+	+	+
L-Malate utilized	+	+	+	+	+
DNase produced	-	+/-	-	-	-

Symbols: the +/- symbol indicates that a majority of species are positive; -/+ symbol indicates that a majority of species are negative.



## Preservation

*Erwinia* and its related genera can be stored indefinitely in the lyophilized state. Cells in exponential phase of growth are harvested by centrifugation and resuspended in 1% sterile solution of powdered milk (Carnation brand). An appropriate aliquot of the mixture is placed in an ampoule and is quickly frozen in liquid nitrogen or in ethanol containing chunks of solid CO<sub>2</sub>. The frozen mixture is lyophilized under vacuum until it is completely desiccated. The ampoule is sealed in vacuo with a torch, appropriately labelled, and stored in a cold room at temperatures between 4 and 16°C. For routine use, it may be convenient to store cultures in the frozen state. In this case, bacterial cells are resuspended in 1% sterile solution of powdered milk prepared in 10% glycerol. The mixture is stored at -70°C. Storing the mixture at -20°C is not recommended. Short-term (1 year maximum) storage of the isolate can rely on specialized media such as Preservation agar (P agar), which contains per liter 5g peptone, 5g sodium chloride, 0.03g cysteine, and 10g agar. The medium is autoclaved in completely filled screw capped tubes. Bacteria are preserved as stab cultures that were allowed to grow for about 4–8 days and then stored at 4°C.

## Genetics

As members of the *Enterobacteriaceae*, *Erwinia*, *Enterobacter*, *Pantoea*, *Pectobacterium* and *Brenneria* are all amenable to genetic manipulation. The molecular genetic tools used in studies dealing with *Escherichia coli* are directly applicable to the study of members of this group of bacteria. Generally the plasmid replicons that contain the origin of DNA replication of the ColE1 plasmid of *E. coli* replicate well in these bacteria. Various nonreplicative plasmids, such as those containing the origin of DNA replication of plasmid R6K (which requires an accessory initiator protein like  $\pi$  for replication), have been used to deliver transposable elements to generate mutants. Essentially, the vector harboring the R6K origin is unable to replicate in the recipient bacteria, thus leaving behind the transposon that jumped onto the chromosome of the recipient. Transposon mutagenesis has opened the way to identify genes involved in various metabolic and physiological pathways in these bacteria. When the bacterium of interest is not amenable to transposon mutagenesis, chemical mutagenesis using nitrosamine and alkane sulfonic esters, such as nitrosoguanidine and ethyl methane sulfonate, respectively, have been used. Chemically derived mutants defective in the phenotype of interest

would be identified in the affected gene by genetic complementation using a genomic library of the parental strain. Besides the generation of mutants, chromosomal genes can be mobilized by a transducing phage if available. The transducing bacteriophage  $\pi$ M1 was used to transfer chromosomal markers in *Erwinia carotovora* subsp. *atroseptica* (Toth et al., 1997). Because of their economic importance, a number of genes involved in pathogenicity and virulence have been identified in *Erwinia* generic group members. The general approach has been to generate mutants affected in a given phenotype, followed by the isolation of the gene or genes responsible for the phenotype, and characterization of the product or products made by the gene or genes of interest. Hence, this approach has been used to identify the genes (avirulence genes) involved in eliciting the hypersensitivity response in non-host plants. The hypersensitive response-pathogenicity or *hrp* genes have been identified in several necrogenic strains of this group of bacteria.

## Epidemiology

The disease cycle of this group of bacteria can be classified according to the way they infect and cause disease. For the necrogenic group of pathogens, there are good examples of insect transmission of the pathogen generally infecting at the early blossom stage of the host plant. For example, the fire blight pathogen *Erwinia amylovora* is transmitted primarily by the honeybee that becomes contaminated or infested by this bacterium during visits to blossoms and oozing sap of an infected tree. *Enterobacter cloacae*, the cause of the internal yellowing disease of papaya fruit, is transmitted by the oriental fruit fly (Nishijima et al., 1987). The dissemination of pink disease appears to be by fruit flies, since timely applications of insecticides dramatically reduces the incidence of this disease. Although transmission of necrogenic bacteria is vectored efficiently by insects, cultural practices also facilitate the spread of this group of bacteria. For example, the mechanical harvester transmits *Erwinia rubrifaciens* (emended as *Brenneria rubrifaciens*) from diseased walnut trees to healthy trees (Kado and Gardner, 1977). The disease cycle of this group of bacteria originates from diseased material in the form of ooze harboring the inoculum that is picked up either by flying insects or harvesting machinery and transmitted to various portals of entry on the host.

Bacteria of the macerative group, represented by members of the genus *Pectobacterium*, have a disease cycle usually associated with the rhizosphere. Infected plant parts tilled into the soil

serve as reservoirs for bacteria belonging to this group. For example, *Pectobacterium carotovora* subsp. *carotovora* is known to infect various tuber, corn, and bulbs of vegetable and ornamental crops. Replanting in soils containing the infected plant parts exposes the new seedlings, seed pieces, etc., to resident macerative bacteria like *P. carotovora* subsp. *carotovora*. Wounds caused by harvesting equipment allow entry of the organism into host tissues where infection is initiated. Microaerophilic and anaerobic conditions created by packing the harvested crop into bins or rail cars are most conducive for soft rot. The commercial processing of vegetables that usually are washed in large baths can become contaminated by the bath water used to wash a diseased tuber, root, etc. Dressing water often becomes contaminated with these bacteria and any resulting wounds made during the vegetable washing process are usually sites for postharvest infection that is usually seen at the produce processing station in supermarkets.

Members of the vascular wilt group infect their host plant systemically. It is thought that because organisms can multiply quickly and elaborate exopolysaccharide materials in the vascular system, they cause occlusion of the vessel elements culminating in dieback and wilting symptoms. The disease cycle of this group of pathogens, typified by *Pantoea stewartii* subsp. *stewartii*, includes the transmission of the bacterium by an insect vector. In the case of *P. stewartii* subsp. *stewartii*, the corn flea beetle (*Chaetocnema publicaria*) disseminates the pathogenic bacterium in cornfields. *Erwinia tracheiphila*, which causes bacterial wilt of cucumber and related bacteria, is spread via the striped cucumber beetle (*Acalymma vittata*) and the spotted cucumber beetle (*Diabrotica undecimpunctata*). Interestingly, these bacteria overwinter in the insect.

The tumorigenic group is best represented by *Pantoea agglomerans* pv. *gypsophilae*, which induces tumors or galls on *Gypsophilum* (Baby's Breath). The source of inoculum is primarily from diseased plants bearing galls or nodules. Infected plant stocks, usually originating from a nursery that does not follow sanitation practices, spreads this organism.

## Disease

Members of the genus *Erwinia*, *Enterobacter*, *Pantoea*, *Pectobacterium*, or *Brenneria* are associated with plants, and many are pathogens of specific plant species. For example, *Erwinia amylovora* favors plants of the Rosaceae family (apple, pears, quince), whereas *Pantoea stewartii* subsp. *stewartii* infects plants of the maize family

specifically *Zea mays* L. (sweet corn); and *Brenneria rubrifaciens* infects specifically walnut trees of the species *Juglans regia*, with *J. regia* cv. *Hartley* being the most susceptible commercial cultivated variety. Hence, pathogens of these five genera display host specificity, even though species of the genus *Pectobacterium* infect a relatively wide range of tuber-, corn- and bulb-crops.

Host specificity is conferred by specific residence-establishing genes, defined herein as "res" genes and is assisted by the products of the *hrp* genes (for hypersensitive response pathogenicity). *Res* genes play an important role in maintaining ecological fitness (either in the rhizosphere or phyllosphere) of the pathogen. The importance of *res* genes in conjunction with *hrp* (or *hrp*-equivalent genes) genes is exemplified in other bacterial systems such as with *Agrobacterium tumefaciens*, where an isoflavonoid-inducible efflux pump is essential for competitive colonization of roots (Palumbo et al., 1998); with *Pseudomonas syringae* pv. *syringae*, where certain *hrp* genes (*hrpJ* and *hrpC*) are needed for epiphytic growth (Hirano et al., 1999; Yu et al., 1999); and with *Xanthomonas campestris* pv. *campestris*, where the *hrpXc* regulatory gene was found essential for colonizing cauliflower plants (Kamoun and Kado, 1990) and is highly conserved among all *Xanthomonas* pathovars (Oku et al., 1998).

The combined activities of the *res* gene and *hrp* genes, which are involved in the secretion and injection of pathogenicity factors via a Type III secretion system into the cells of the host plant, are essential for establishing epiphytic fitness. The functional role of the *res* and *hrp* genes are not mutually exclusive because the effectors, which are encoded by the *res* and *hrp* genes, require the Type III secretion system, which is also encoded by *hrp* genes and injected into the host cell by the bacterial pathogen. The Type III secretion system is encoded by a cluster of genes contained within a pathogenicity island. More than one Type III secretion system may exist in a pathogen, with each being encoded by separate pathogenicity islands. For example, two distinct virulence-associated Type III secretion systems are encoded within pathogenicity islands, SPI1 and SPI2, of *Salmonella* spp. (Hensel et al., 1997; Galán and Collmer, 1999). The Type III secretory pathway involves approximately 20 proteins that carry exoproteins directly from the cytoplasm to the cell surface, from where they are then injected into an animal or plant cell upon contact (depending whether the member of the *Enterobacteriaceae* is a mammalian or plant pathogen).

The Type III secretion system and the effector proteins are highly conserved and are related to those in other members of the *Enterobacteriaceae* such as *Yersinia*, *Salmonella*, and *Shigella*

(Galán and Collmer, 1999). The conservation of the Type III secretion apparatus and most of the effector proteins reflect the conservation of the genes of the pathogenicity island that was likely acquired by horizontal gene transfer. This is supported by the presence of homologs of genes in the *hrp* pathogenicity island in *Erwinia amylovora* to those in *Ralstonia solanacearum*, *Xanthomonas campestris* pv. *campestris*, *Pseudomonas syringae* pv. *syringae*, *Yersinia enterocolitica*, *Shigella flexneri*, and *Salmonella typhimurium* (Bogdanove et al., 1996). Thus, Type III secretion systems are functionally conserved as well. For example, the Type III secretion system of *X. campestris* pv. *vesicatoria* is able to secrete PopA of the Hrp system in *Ralstonia solanacearum*; AvirB of *Pseudomonas syringae* pv. *glycinea*, and the cytotoxin YopE of *Yersinia pseudotuberculosis* (Rossier et al., 1999).

The overall role of the Type III secretion system is to deliver virulence and pathogenicity effectors that establish and maintain infection and cause disease. Loss of this delivery system's function results in an avirulent phenotype and the inability to compete effectively in the infection court, defined here as the site of infection whether it be on the roots or foliage of the plant. Pathogenicity of the *Erwinia* group of pathogens, therefore, is highly dependent on the Type III secretion system. Because the genes of the Type III system are highly conserved, these pathogens are likely to have originated through gene acquisition via a horizontal gene transfer system.

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## The Genera *Photorhabdus* and *Xenorhabdus*

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### Introduction

The genera *Photorhabdus* and *Xenorhabdus* are members of the family Enterobacteriaceae that encompass the intestinal bacterial symbionts living in commensalism with entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema*, respectively (Akhurst and Boemare, 1990; Forst et al., 1997). Most of them are pathogenic for insects when injected into the hemocoel. In addition, some nonsymbiotic strains of *Photorhabdus* have been identified as opportunistic pathogens for humans (Farmer et al., 1989; Peel et al., 1999). Various insect and vertebrate symbionts are members of the  $\gamma$ -subclass of Proteobacteria, which contains a wide spectrum of animal and human pathogens, such as members of the families Enterobacteriaceae, Legionellaceae, Pasteurellaceae, Vibrionaceae, and the genera *Pseudomonas* (sensu stricto) and *Acinetobacter* (Stackebrandt, 1999). Symbionts of EPNs are phylogenetic neighbors of an important group of endosymbionts of insects (the RDP tree of the {Bergey's Web site}). Most of the insect endosymbionts cluster according to the phylogeny of their eukaryotic hosts, which may indicate coevolutionary events (Stackebrandt, 1999). A similar cospeciation (See Habitat/Cospeciation in this Chapter) also was observed in the case of EPNs (Akhurst, 1983; Boemare et al., 1997a; Forst et al., 1997).

However, though most of the insect symbionts are endocytobionts and not cultivable, *Xenorhabdus* and *Photorhabdus* have the distinct advantage of cultivability on standard bacteriological media. Moreover, their hosts can be easily axenized. Consequently, pure cultures of axenic nematodes and bacteria are available for combination in gnotobiological experiments that demonstrate the viability and performance (both qualitative and quantitative) of the bacterial/helminthic symbioses (Boemare et al., 1997a). Other bacterial–animal symbioses involving cultivable Proteobacteriaceae have been described: some marine animal light-organ symbionts (Nealson et al., 1990) such as the model *Vibrio*

*fischeri* (associated with the squid *Euprymna scolopes*) and *Aeromonas veronii* bv. *sobria* (symbiont of the medicinal leech *Hirudo medicinalis*). In this latter, despite the diverse bacterial flora found along the tegument of the leech, the *A. veroni* bv. *sobria* are typically found as a pure culture in the gut, suggesting that these symbionts possess a special capability that enables them to proliferate in the medicinal leech (Graf, 2000). *Vibrio harveyi*, often commensal with marine animals, may be pathogenic for the black tiger prawn, *Penaeus monodon* (Manefield et al., 2000), as are the terrestrial luminous *Photorhabdus* for insects.

### Taxonomy

Initially only one genus, *Xenorhabdus*, was described as encompassing all the symbionts of the entomopathogenic nematodes. Those strains initially considered as belonging to the species *Xenorhabdus luminescens* clearly form a DNA-relatedness group that is distinct from all the other *Xenorhabdus* strains (Akhurst et al., 1996; Boemare et al., 1993). These DNA data, together with the significant differences in phenotypic characters between “*X. luminescens*” and the other *Xenorhabdus* species (Akhurst and Boemare, 1988; Boemare and Akhurst, 1988), differences in fatty acid composition (Janse and Smits, 1990), and chemotaxonomic data (Suzuki et al., 1990) led to the transfer of *X. luminescens* into a new genus, *Photorhabdus*, as *Photorhabdus luminescens* comb. nov. (Boemare et al., 1993). Nevertheless, comparison of 16S rDNA sequences of the type strains of *Photorhabdus* and *Xenorhabdus* species indicate the close phylogenetic relationship of these two genera (Rainey et al., 1995). The Ribosomal Dataset Project (RDP) tree established that the two genera branch deeply in the family Enterobacteriaceae without any common ancestor. They are neighbors of *Proteus vulgaris* and *Arsenophonus nasoniae*, and between *Salmonella*, *Erwinia*, *Serratia*, and several endosymbionts of insects (*Hafnia*, *Rahnella* and *Yersinia*; Liu et al., 1997;

the RDP tree of the {Bergey's Web site}). All *Xenorhabdus* strains could be clearly distinguished from all strains of *Photorhabdus* by the 16S rDNA signature sequences; *Xenorhabdus* have a TTTCG sequence at positions 208–211 (*E. coli* numbering) of the 16S rDNA, whereas *Photorhabdus* have a TGAAAG sequence at positions 208–213 (Szállás et al., 1997).

There are also biological arguments to distinguish these two genera. In all the microbial ecological surveys undertaken in the five continents (more than 100 independent reports), a *Xenorhabdus* isolate has never been recovered from *Heterorhabditis* or a *Photorhabdus* from *Steinernema*. Moreover, by testing the specific interaction with the nematode host, gnotobiological experiments demonstrate that *Photorhabdus* isolates do not support culture of any *Steinernema* species in vitro (Akhurst, 1983), although in some combinations, they support culture of non-host *Heterorhabditis* spp. (Akhurst and Boemare, 1990; Han et al., 1990). Similarly *Xenorhabdus* spp. do not support culture of *Heterorhabditis* (Akhurst, 1983).

For identifying the diversity of the *Photorhabdus* and *Xenorhabdus*, a fast method using the polymorphism of the 16S rRNA genes was described by Brunel et al. (1997). Fischer-Le Saux et al. (1998) applied this polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method (Fig. 1), which gives an excellent estimation of the diversity of the genus by using efficient restriction endonucleases after first checking that they provide the most discriminative restriction patterns within the given sample. It allows analysis of a large sample of different isolates giving a series of genotypes, each one corresponding to a specific restriction pattern. Based on the total amplified sequence of the 16S ribosomal RNA genes, the method provides reference restriction patterns for identifying other isolates.

According to rule 65 (2) of the *International Code of Nomenclature of Bacteria*, generic and subgeneric names, which are modern compounds of two or more Latin or Greek words, have the gender of the Greek or Latin word used as the last component of the compound. Consequently, *Photorhabdus* and *Xenorhabdus* (genera with names ending in *rhabdus* [from *rhabdos*, the Greek word for rod] are feminine) become in modern Latin feminine words, explaining the feminine species and subspecies names when they refer to adjectives (Euzéby and Boemare, 2000). Thus, “*X. nematophilus*” and “*X. japonicus*” become *X. nematophila* and *X. japonica*, and the new species of *Photorhabdus*, *P. temperata* and *P. asymbiotica*. Otherwise when the species names refer to an author, they take the gender of the author, such as *X. bovienii*, *X.*

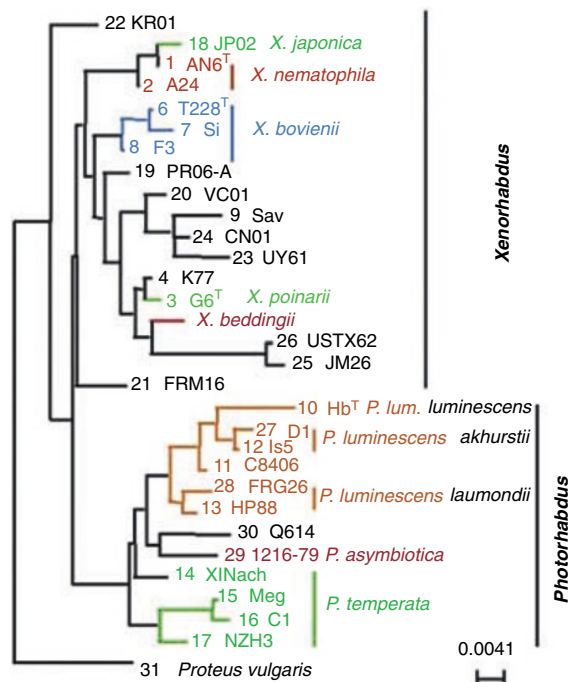


Fig. 1. Phylogenetic tree of PCR-RFLP 16S rDNA genotypes *Xenorhabdus* and *Photorhabdus*. The neighbor-joining method was applied to 30 defined genotypes, from a total of 117 strains. The number of the genotype according to Fischer-Le Saux et al. (1998) is followed by the name of the representative strain.

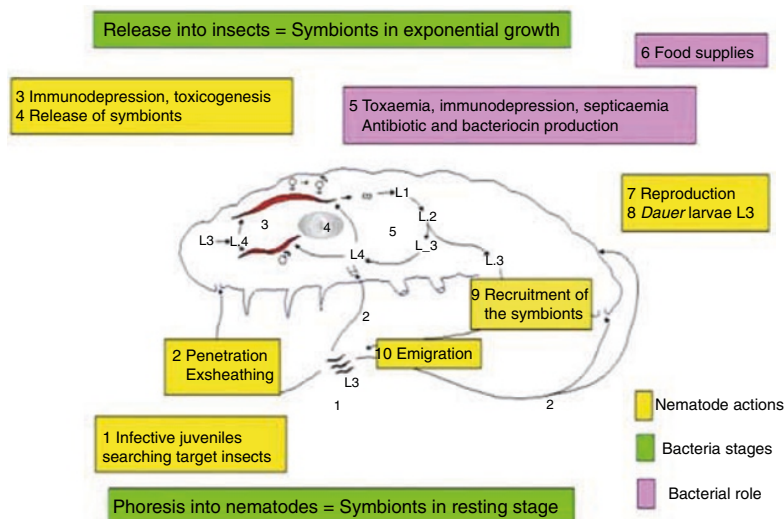
*poinarii*, *X. beddingii*, *P. luminescens* subsp. *laumondii* and *P. luminescens* subsp. *akhurstii*.

## Habitat

### Life Cycle

Symbionts of EPNs live in two different habitats during their life cycle: they survive in the gut of the free-living stage of their nematode host, and they are inoculated into and multiply in the body cavity of insects, thus creating a pure monoxenic culture (See Habitat and Ecology sections of the chapters *Photorhabdus* and *Xenorhabdus*). So the life cycle of the EPN symbionts is unique because it involves both symbiosis of nematodes and pathogenesis of insects, as summarized in Fig. 2. The infective juveniles (also named “dauer” larvae), which are the nematodes’ third stage, search (cruiser species) or wait (ambusher species; Gaugler, 1993) for an insect prey (step 1). When the target insect is found they penetrate by natural openings (mouth, anus, spiracles) or directly through the tegument (mainly *Heterorhabditis*). During penetration, the nematodes exsheath the second stage cuticle, which was retained during their period in the soil, before

Fig. 2. Life cycle of entomopathogenic nematodes and their bacterial symbionts.



entering into the body cavity of the insects (step 2). They transform into the fourth and adult stages and during this time induce a toxicogenesis (Boemare et al., 1983b; Boemare et al., 1982), releasing an immune depressive factor (step 3) active against antimicrobial peptides produced by the insects (Götz et al., 1981). This immune depressive factor is presumably a protease that facilitates release of the nematode's bacterial symbionts (step 4). The bacteria multiply, and at the final stage, the insects die due to the septicemia (step 5). Depending on the insect, nematode and bacterial symbionts, the pathogenic process can be the result of the action of one partner or both together (see *Xenorhabdus*/Ecology in this Chapter). Sometimes a toxemia induced by the symbiont precedes the resulting septicemia (Forst et al., 1997). At the end of the bacterial multiplication, production of a large variety of antimicrobial compounds (see *Photorhabdus* and *Xenorhabdus* in this Chapter) prevents microbial contamination, mainly from the insect intestinal microflora. By using the food supplies provided by the bacterial biomass and the metabolized insect tissues (step 6), the nematodes reproduce in the insect cadaver (step 7) for one, two or three generations. Thus, bacteria create suitable conditions for the development of their nematode host in the insect cadaver. At the end of the parasitism, the recruitment of some bacterial cells by the dauer larvae of the nematodes (step 9), before leaving the insect (step 10), maintains the perennality of the symbiosis through the generations.

## Physiology

### Phase Variation

A highly significant feature of bacteria of the genera *Photorhabdus* and *Xenorhabdus* is a

phase variation that occurs during the stationary period of the growth cycle (Akhurst, 1980). It can be easily detected by two major properties: dye adsorption and antibiotic production. Only phase I of the symbionts has been detected in nature, but under in vitro conditions, a variable proportion undergoes profound change affecting colony and cell morphologies, motility, secondary metabolites, endo- and exo-enzymes (including respiratory enzymes; Akhurst, 1980; Boemare and Akhurst, 1988; Givaudan et al., 1995; Smigielski et al., 1994b). The timing and extent of the phase change is largely unpredictable, but the rate of change from phase I to phase II is generally greater than the reverse. In contrast to *Photorhabdus* where no reversion has yet been reported, the revertants obtained with *X. nematophila* (Akhurst and Boemare, 1990; Givaudan et al., 1995) indicate that the variation in the genus *Xenorhabdus* better fits the classical definition of phase variation. Indeed, phase variation in bacteriology is commonly accepted as a reversible genetic event usually mediated by DNA instability such as DNA inversion. Usually such a phenomenon affects one or a small number of gene products and is reversible at a significant frequency. In the case of *Photorhabdus*, many phenotypic traits are altered in phase II variants and no reversion has been confirmed. Some described "revertants" came from intermediate variants (Krasomil-Osterfeld, 1995), but not from true phase II variants, which have a complete loss of phase I phenotypic traits. Consequently the use of the phrase "phase variation" for *Photorhabdus* phenotypic shifts is not exactly appropriate, but is commonly used to refer to an alternative balance in expression/nonexpression of physiological traits from the same genome.

In general, variation in phase-related characters of *Photorhabdus* and *Xenorhabdus* has been

Table 1. Phase characters of *Photorhabdus* and *Xenorhabdus*.

	Phase I variants	Phase II variants
Colonial characteristics		
— Morphology	mucoïd	smooth
— Stickiness	+	—
— Dye adsorption	+	—
— Pigmentation	+	— or different <sup>1</sup>
— Motility	+ <sup>2</sup>	—
— Swarming	+ <sup>2</sup>	—
Ultrastructural elements and cytological properties		
— Protoplasmic crystalline inclusions	+	d
— Flagella	+ <sup>2</sup>	—
— OpnA, CpnB	+ <sup>2</sup>	—
— Fimbriae (or pili)	+ <sup>2</sup>	—
— Glycocalyx thickness	+	w
— Insect hemocyte agglutination	+	—
— Erythrocyte agglutination	+	—
Enzymatic activities and secondary metabolites		
— Respiratory enzymatic activity	w	+
— Bioluminescence	+ <sup>1</sup>	w
— Antibiotic production	+	d
— Lecithinase	+	—
— Tween-esterases	d	+
— Proteases	+	d

reported qualitatively (“+” and “–”). However, for every character that can be quantified (e.g., luminescence and antibiotic production), it is clear that the difference between phases is a matter of magnitude, not presence or absence. It is highly probable that this holds true for all phase-related characters.

Table 1 summarizes the characters affected by phase variation. The bacterium isolated from the infective stage (dauer larvae) was named the phase I variant (Boemare and Akhurst, 1988). Phase I colonies are mucoïd and stick to the loop when streaked on plates, produce antibiotic molecules (Akhurst, 1982a), adsorb dyes when incorporated into agar (e.g., the neutral red in MacConkey agar). Phase II appears spontaneously during stationary growth period from in vitro culture and during nematode rearing on artificial diets. Phase II colonies are not mucoïd, do not adsorb dye and do not produce antibiotics.

Numerical analyses of phenotypic data for the two phase variants of multiple *Xenorhabdus* and *Photorhabdus* strains correspond to the genotypic definition of species (Fig. 3). However, these analyses also show that phase variation is a general phenomenon throughout the genera that results in conspecific strains grouping by phase status before grouping by species (compare Fig. 3a with Fig. 3b, where some phase-related characters are deleted from the analysis). Phase variation is a general phenomenon at the infra-specific level for both genera. It does not affect the phenotypic clustering of the species, although phase variants are more similar inside

each species. However, depending on the phase characters affected for each strain, clustering by phase variants or by strains may be different. Thus, some strains have phase variants differentiated by many characters (e.g., strains F1 and A24 of *X. nematophila*, strains Q614 and HI of *Photorhabdus*; Fig. 3b), others have only a few characters distinguishing the two phase variants (strains SK2 of *X. bovienii* and NC116 of *X. nematophila*; Fig. 3a).

The variation between phases in phenotypic data has been the source of some discrepancies between results published by different laboratories. Consequently, before starting the analysis of such phenotypic characters, we have to recommend an accurate screening of clones. Thus, for a taxonomic study, the two extreme clones of each strain expressing the largest number of different phenotypic responses to bacteriological tests, designated as phase I and phase II variants, must be selected. Clones that are intermediate (i.e., expressing some phase I and some phase II characters) should be eliminated from a taxonomic study, particularly for the genus *Photorhabdus*. Practically, before any mathematical treatment devoted to the taxonomy of species, the physiological data is compiled to check any positive response of related clones to a strain, and to be consistent with the definition of all the potentialities of the given strain. This is also a reason to take great care to harvest isolates expressing all the strain properties during the isolation from the nematode host. Conversely, these intermediate expressions are of great interest for genetic studies.



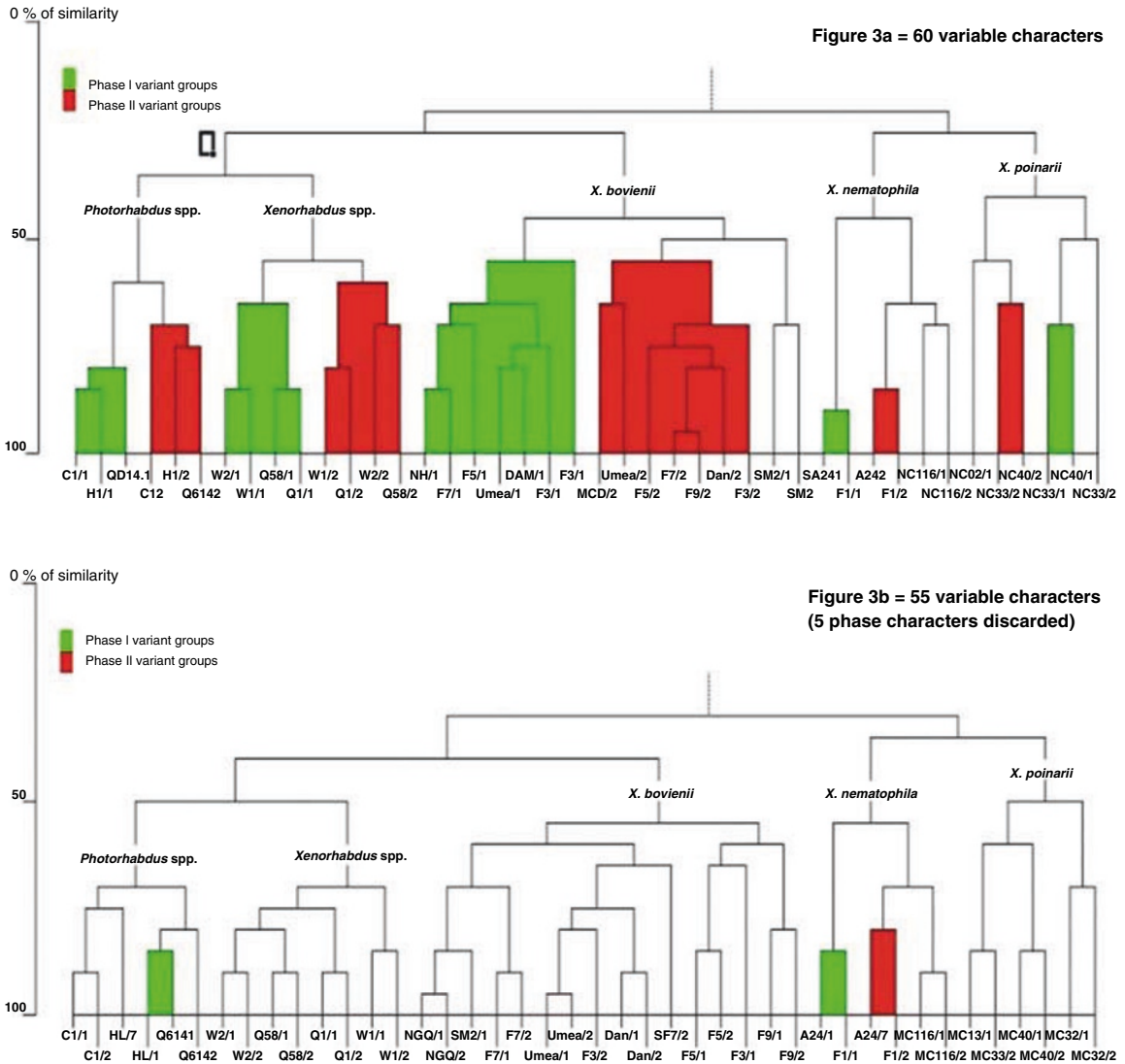


Fig. 3. Numerical analysis of phenotypic data from *Xenorhabdus* and *Photorhabdus* phase variants. All the data are treated as two-state (0,1) characters. Characters that were positive or negative for all isolates (i.e., that were not discriminative) were deleted (180/240). Similarity coefficients were calculated with the Jaccard coefficient (Sneath and Sokal, 1973). To construct dendrograms, the internode lengths were calculated for each coefficient by complete linkage clustering (furthest-neighbor sorting strategy: FNS) using the GENSTAT program (Alvey et al., 1980). However, simple linkage (nearest-neighbor clustering), group-average sorting (UPGMA), weighted clustering (WPGMA), unweighted and weighted centroid clusterings were also tested (not shown) and gave comparable results. The dendrograms obtained with FNS best illustrated the universality of the phase variation concept occurring in both genera. Codification of isolates: N/1 = phase I variant of strain N; N/2 = phase II variant of strain N. (a) Dendrogram obtained with the 60 characters that were not common to both phases of all strains. Note that for no strain, except *X. bovienii* strain SK2 and *X. nematophila* strain NC116 where phase characters are poorly expressed, did the two phase variants cluster together before clustering with another strain. (b) Dendrogram obtained from the same data set with 55 characters, the 60 previous characters minus 5 identified as being the principal phase-related characters. They were: antibiotics, dye-binding, phospholipase, protoplasmic inclusions, pigmentation. When these phase-related characters were deleted from the analysis, both phase variants of each strain clustered together before clustering with another strain (except in *Photorhabdus* spp. and *X. nematophila*, groups where some strains expressed additional phase-related characters). Updated from data published by Akhurst and Boemare (1988).

Phase I variants provide and protect essential nutrients for the nematodes by killing and metabolizing the insect host and producing a range of antimicrobial agents. Although phase II variants may also kill the insect host and are

capable of colonizing the dauer vesicle of *Steinernema* or the anterior part of the intestine of *Heterorhabditis*, they are less effective in providing growth conditions for the nematodes (Akhurst, 1980; Akhurst, 1982a; Akhurst and

Boemare, 1990) and have never been found associated with naturally occurring nematodes. In addition some *Photorhabdus* phase II variants may be deleterious to their original *Heterorhabditis* (Ehlers et al., 1990).

### Convergence

*Photorhabdus-Heterorhabditis* and *Xenorhabdus-Steinernema* symbioses are widely divergent. These symbioses are believed to use different mechanisms to maintain superficially similar associations. The similarities in their patterns of infectivity, life cycle and mutualism with nematode should be considered to result from evolutionary convergence. Indeed the symbiotic, pathogenic, and phase variation properties, which are the conditions for such associations (Boemare et al., 1997a), do not necessarily imply the same physiological mechanisms. Information gained from current genetic studies will probably clarify the picture of and help explain the convergent evolution of *Photorhabdus-Heterorhabditis* and *Xenorhabdus-Steinernema* symbioses (Boemare and Akhurst, 1994).

### Applications

These bacteria are now recognized as potentially having great importance beyond their associations with nematodes (See Applications sections of *Photorhabdus* and *Xenorhabdus*). Recently, the use of *Photorhabdus* and *Xenorhabdus* genes encoding for entomotoxins to create transgenic plants for crop protection was proposed (French-Constant and Bowen, 1999). The particular properties of these bacteria in fundamental research terms (such as cellular exportation, exoenzymatic activities, production of many special metabolites, pathogenic processes, ability to differentiate into multicellular populations and colonize different micro-niches) were inducements for the Pasteur Institute and INRA (France) to sequence the whole genome of *Photorhabdus*, which will soon be available to the international community (Kunst et al., 2000; the [http://www.pasteur.fr/recherche/unites/gmp/Gmp\\_projects.html#pl](http://www.pasteur.fr/recherche/unites/gmp/Gmp_projects.html#pl) {Pasteur Web}). We believe that these two bacterial genera will provide in the coming years new insights for microbiology in terms of genetics and physiology, particularly on pathogenic mechanisms and specific metabolic pathways of prokaryote-eukaryote interaction. Presumably researchers also will be able to address shortly an evolutionary history for these mechanisms.

## Genus *Photorhabdus*

### Phylogeny

Only 16S rRNA gene sequence data are available for analyzing the phylogeny of *Photorhabdus*. However, these provide solid trees regardless of the clustering method used (neighbor joining, parsimony or maximum likelihood) for understanding the position of the genus in the radiation of the Enterobacteriaceae. Thus, 16S rDNA sequences from representative strains of *Photorhabdus* (Fischer-Le Saux et al., 1999b; Szállás et al., 1997) were compared to homologous sequences of several species of Enterobacteriaceae. Binary similarity values determined for *Photorhabdus* ranged above 96%, whereas the similarity values for *Photorhabdus* compared to *Xenorhabdus* and other Enterobacteriaceae strains were between 94–96% and 92–95%, respectively. PCR-RFLP analysis (Fischer-Le Saux et al., 1998) and sequence analysis (Fischer-Le Saux et al., 1999b; Szállás et al., 1997; Fig. 4) also verified the existence of three major 16S rRNA clusters that corresponded to the DNA-DNA relatedness groups (see Taxonomy in this Chapter). Depending on the out-group inserted and on dispersion of other related bacteria (Stackebrandt et al., 1997; Stackebrandt and Goebel, 1994), the *P. luminescens* subsp. *luminescens* may aggregate differently with other subgroups or even with *P. asymbiotica*. Nevertheless, sequence analyses of 16S rDNA show that all *Photorhabdus* strains branch deeply within the radiation of the family Enterobacteriaceae, and have a specific TGAAAG sequence at positions 208–213 (*E. coli* numbering). The nearest phylogenetic neighbor as demonstrated by PCR-RFLP (Brunel et al., 1997) and sequencing of 16S rRNA (Fischer-Le Saux et al., 1999b; Rainey et al., 1995; Suzuki et al., 1996; Szállás et al., 1997) is *Xenorhabdus*, then *Proteus*.

It appears that the genus *Photorhabdus* evolved after the main radiation of *Xenorhabdus* species occurred (Forst et al., 1997). This hypothesis, originally derived from the analysis of only seven strains (Rainey et al., 1995), was verified when the 16S rDNA analysis was extended to a balanced number of datasets from *Photorhabdus* and *Xenorhabdus* in comparison to other genera and families (Fischer-Le Saux et al., 1999b; Suzuki et al., 1996; Szállás et al., 1997).

### Taxonomy

NOMENCLATURE AND FAMILY LINKING Poinar et al. (1977) isolated a bacterium from a previously unknown nematode, *Heterorhabditis* bacteriophora (Poinar, 1976), which was apparently similar to a *Xenorhabdus* sp. but noticeably

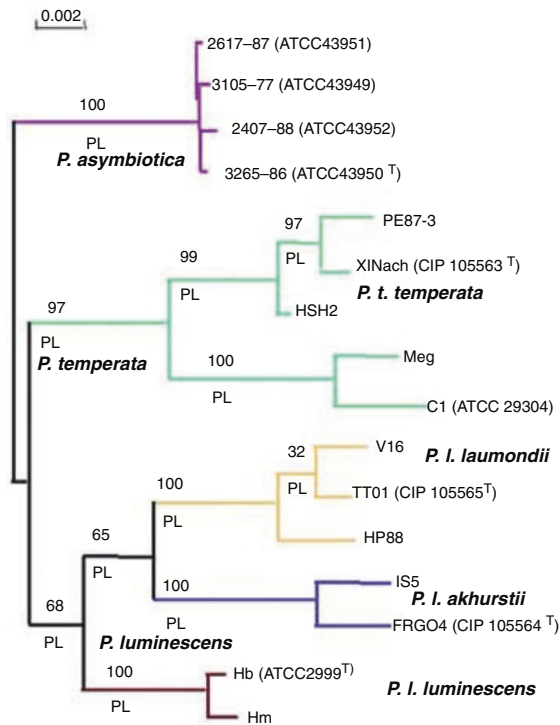


Fig. 4. Phylogenetic tree of *Photorhabdus* spp. from 16S rDNA sequences obtained by the neighbor-joining method (Saitou and Nei, 1987) using a bootstrap approach (Felsenstein, 1985) to determine the reliability of the topology obtained (numbers given above the nodes). Those clusters also obtained by parsimony (Kluge and Farris, 1969) and by maximum likelihood (Felsenstein, 1981) are indicated by a "P" and an "L" below the nodes, respectively. The accession numbers are given after the strain names. Internode values are only weak at the linkage of both subspecies *laumondii* and *akhurstii* with *luminescens*. Here no out-group has been inserted in the analysis to establish the diversity inside the group of *Photorhabdus*. Depending on the out-group inserted and on dispersion of other related bacteria (Stackebrandt et al., 1997; Stackebrandt and Goebel, 1994), the *P. luminescens* subsp. *luminescens* may aggregate differently with other subgroups or even with *P. asymbiotica*. Such proximity can be also observed by phenotypic analysis (see Fig. 6). The bar indicates a distance of 0.002 substitutions per site (s/s). (Modified from Fischer-Le Saux et al., 1999b).

luminous. They proposed inclusion of this luminous species in the genus as *X. luminescens* (Thomas and Poinar, 1979). However, the genus *Photorhabdus* was created in 1993 to accommodate this species as *Photorhabdus luminescens* comb. nov. (Boemare et al., 1993). Thus, due to the rules of bacterial nomenclature, the type species was named *Photorhabdus luminescens*, creating an unavoidable pleonasm.

By DNA/DNA hybridization, *Photorhabdus* is only 4% related to *Escherichia coli*, the type species of the type genus for the family. However, *Photorhabdus* possesses the enterobacterial common antigen (Ramia et al., 1982). These data

indicate that, although most biochemical tests used to differentiate the Enterobacteriaceae are negative for *Photorhabdus* and although this genus is only distantly related to the core genera of this family (Farmer, 1984), *Photorhabdus* should be retained in the Enterobacteriaceae. At present time the genus includes three species with four subspecies (Boemare and Akhurst, 2000; Fischer-Le Saux et al., 1999b).

**THE POLYPHASIC APPROACH TO SPECIES DELINEATION** Bacteriologists cannot delineate species by crossbreeding, a method used by other biologists, but rather by comparing the total DNA of strains hypothesized to be closely related. Indeed, DNA-DNA hybridization is the key method to delineate bacterial species inside a well-distinguished group (Wayne et al., 1987). Consequently a species in bacteriology is an artificial concept. It is defined as a group of strains sharing approximately 70% or greater DNA/DNA relatedness associated with 5°C or less change in melting temperature ( $\delta T_m$ ). However, bacterial taxonomists recognize that these values are indicative rather than absolute (Vandamme et al., 1996). Based on data obtained using the S1 nuclease method, Grimont (1980) made the definition more robust by stating that strains having 80% reassociation with  $\delta T_m$  below 5°C did belong and those having less than 60% reassociation with  $\delta T_m$  more than 7°C did not belong to the same species. For reassociation values between 60 and 80%, or  $\delta T_m$  values between 5 and 7°C, strains should be carefully studied to delineate species.

Thus, by using the hydroxyapatite (HA) method to analyze DNA-DNA *Photorhabdus* heteroduplexes, two DNA-relatedness groups associated with nematode *Heterorhabditis* and one containing strains found in human clinical specimens (Farmer et al., 1989) were recognized (Akhurst et al., 1996). Recently, restriction patterns obtained after amplification of the 16S rDNAs allowed identification of 12 genotypes among *Photorhabdus* strains (Fischer-Le Saux et al., 1998). By hybridizing the DNA of some representative strains of each ribosomal genotype, and by using the S1 nuclease method, the previous three genomic groups were confirmed (Fischer-Le Saux et al., 1999b; Fig. 5). They exhibited between them DNA-DNA hybridization values lower than 42% with  $\delta T_m$  higher than 8.7°C. As reported before (Grimont et al., 1980), DNA reassociation values obtained with the S1 method were lower than those obtained with the HA method. Moreover, the phylogenetic trees inferred from the complete 16S rDNA sequence analysis (using neighbor joining, parsimony and maximum likelihood) delineate the same clusters as both DNA-DNA hybridization

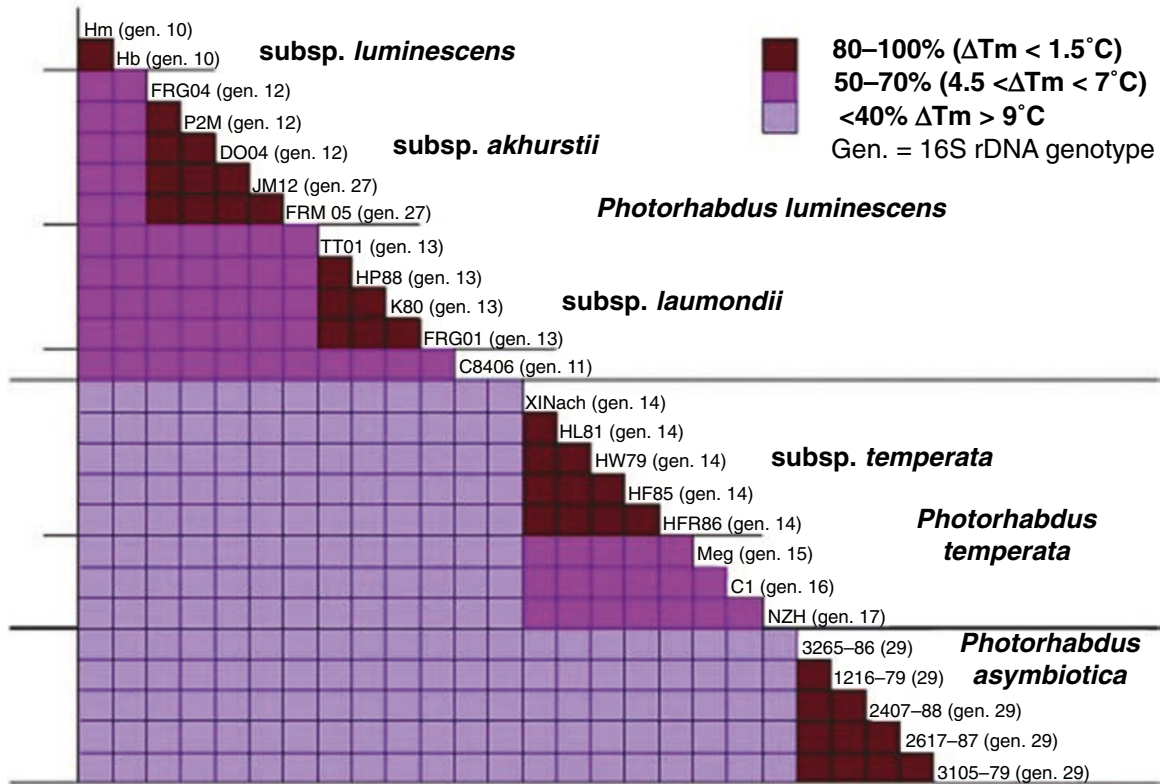


Fig. 5. Interpretation of the DNA/DNA binding ratio data showing the levels of relatedness of *Photorhabdus* strains. Three clusters of DNA-reassociation values were delineated: (i) higher than 80% associated with  $\delta T_m$  lower than  $1.5^\circ\text{C}$  correspond to relatedness values between members of the same subspecies (dark pink boxes); (ii) 50–70% with  $\delta T_m$   $4.5\text{--}7^\circ\text{C}$  correspond to levels of relatedness occurring among members of different subspecies within a species (pink boxes); and (iii) lower than 40% with  $\delta T_m$  higher than  $9^\circ\text{C}$  correspond to reassociation values between members of different species (mauve boxes). The 16S rRNA genotype designations according to Fischer-Le Saux et al. (1998) are indicated between brackets after the strain names. (Modified from Fischer-Le Saux et al., 1999b.)

methods (Fig. 4). All members of these genomic groups clustered with high bootstrap confidence values.

Clustering of phenotypic data by multivariate correspondence analysis (Fig. 6) shows a good correlation with the previous genomic analyses (Figs. 4 and 5) and helped the taxonomist to recognize discriminative characters between the species. We note for instance (Table 2) that the maximum growth temperature, DNase, urease and indole production, utilization of mannitol and of DL-lactate, and esculin hydrolysis may be specific diagnostic characteristics depending on the species.

Symbols: +, 90–100% of strains are positive; [+], 76–89% are positive; d, 26–75% are positive; [+], 11–25% are positive; =, 0–10% are positive, w (e.g., [+]w), indicates a weak reaction.

Abbreviations of type strains: ATCC, American Type Culture Collection (Rockville, Md.); CIP, Collection de l'Institut Pasteur (Paris, France); <sup>T</sup>, type strain. <sup>a</sup>All tests were done at  $28^\circ\text{C} \pm 1^\circ\text{C}$  unless otherwise noted. <sup>b</sup>Numbering of genotypes

according to Fischer-Le Saux et al. (1998). (Modified from Fischer-Le Saux et al., 1999b.)

Consequently, species and subspecies among *Photorhabdus* were delineated by applying a polyphasic approach, combining 16S rDNA, DNA-DNA hybridization and phenotypic data (Fischer-Le Saux et al., 1999b). The species are: a) *P. luminescens* containing the type strain and the symbiotic strains of ubiquitous and/or warm-region nematodes (max. growth at  $35\text{--}39^\circ\text{C}$ ), b) *P. temperata* containing exclusively the symbiotic strains of temperate region nematodes (max. growth at  $33\text{--}35^\circ\text{C}$ ) and c) *P. asymbiotica* originating from clinical samples from the United States. On the basis of 16S rDNA sequencing, the new Australian clinical strains (Peel et al., 1999) will probably constitute a new species (R.J. Akhurst et al., unpublished observation). The one non-luminous strain (Akhurst and Boemare, 1986c) probably is yet another species (Akhurst et al., 1996).

Within *P. luminescens* and *P. temperata*, subgroups of strains shared very high DNA-DNA hybridization values and  $\delta T_m$ s lower than  $1.5^\circ\text{C}$ ,

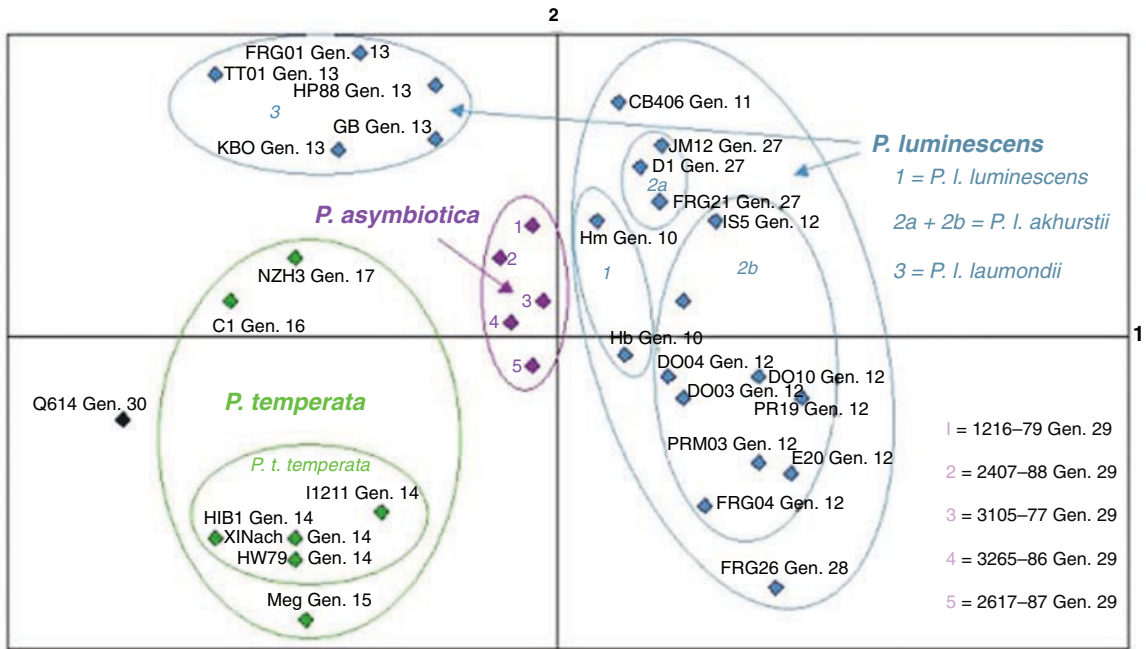


Fig. 6. Multivariate correspondence analysis of phenotypic data from *Photorhabdus* strains. This Q-R reciprocal analysis used the software Statlab (SLP®) taking into consideration only the physiological and biochemical characters scored as variable. The strains are positioned in the A space defined by the two first components. Strains with their PCR-RFLP genotype number (Fischer-Le Saux et al., 1998) are indicated at their position in the mathematical space. The corresponding species and subspecies are encircled. Only one unclassified strain is noted; Q614 (black spot) is a wild-type non-luminous *Photorhabdus* (Akhurst and Boemare, 1986c). Analysis of the inverted matrix (R analysis) shows that the first component is mainly supported by variables of growth temperature and DNase, explaining that the “warm” strains of *P. luminescens* subsp. *luminescens* and *P. luminescens* subsp. *akhurstii* are on the right side of the representation while the “temperate” strains are on the left side. Note that *P. asymbiotica* (pink spots) is a “consensual group” in the middle of the space (see also Fig. 7) near *P. luminescens luminescens* (cf. remark in Fig. 4 legend). Analysis used data published by Fischer-Le Saux et al. (1999b).

and were separated by stable 16S rDNA branching (Figs. 4 and 5). Consequently, *P. luminescens* was divided into three subspecies: *P. luminescens* subsp. *luminescens* (containing the type strain and strains associated with nematodes from the Brecon subgroup of *H. bacteriophora*), *P. luminescens* subsp. *laumondii* (containing strains associated with the HP88 subgroup of *H. bacteriophora*) and *P. luminescens* subsp. *akhurstii* (containing strains associated with *H. indica*). Similarly, a subspecies within *P. temperata* containing strains associated with the Palaearctic subgroup of *H. megidis*, and containing the type strain, was proposed as *P. temperata* subsp. *temperata*. As more strains become available, other subspecies will be defined in *P. temperata* especially for the Nearctic subgroup of *H. megidis*.

**GENUS CHARACTERISTICS** Cells of this genus (Thomas and Poinar, 1979; Boemare et al., 1993) are asporogenous and rod-shaped (0.5–2 by 1–10  $\mu\text{m}$ ). Cell size is highly variable within and between cultures with occasional filaments up to 30  $\mu\text{m}$  long. In the last stage of exponential growth and during the stationary growth period,

spheroplasts may occur with an average of 2.6  $\mu\text{m}$  in size (10–20% of cell population), resulting from the partial disintegration of the cell wall. Proteinaceous protoplasmic inclusions are synthesized inside a high proportion of cells (50–80%) during the stationary period. Cells are Gram negative, motile by means of peritrichous flagella, and facultatively anaerobic, having both a respiratory and a fermentative type of metabolism. Optimum growth temperature is usually ca. 28°C; some strains grow at 37–38°C. Most strains produce pink, red, orange, yellow or green-pigmented colonies on nutrient agar, and especially on rich media (tryptic soy agar, egg yolk agar). Luminosity (usually detectable by the dark-adapted eye) varies in intensity within and between isolates and may only be detectable by a photometer or scintillation counter in some isolates; only one non-luminous isolate is known among approximately 150 reported strains. Spontaneous phase shift occurs in subcultures, inducing the appearance of phase II clones (see Physiology/Phase Variation). The latter are characterized by considerably less neutral red adsorption on MacConkey agar, production of

Table 2. Characteristics that differentiate *Photorhabdus* species.

Species or sub-species	<i>Photorhabdus luminescens</i>	<i>P. luminescens luminescens</i>	<i>P. luminescens laumondii</i>	<i>P. luminescens akhurstii</i>	<i>Photorhabdus temperata</i>	<i>P. temperata temperata</i>	<i>Photorhabdus asymbiotica</i>
Isolated from	<i>Heterorhabditis spp.</i>	<i>Heterorhabditis bacteriophora</i> group Brecon	<i>Heterorhabditis bacteriophora</i> group HP88	<i>Heterorhabditis indica</i>	<i>Heterorhabditis spp.</i>	<i>H. megidis</i> Palaearctic group	Human blood and/or wounds
Type culture collection	ATCC 29999 DSM3368	ATCC 29999 DSM3368	CIP 105565	CIP 105564	CIP 105563	CIP 105563	ATCC 43950
PCR-RFLP 16S rDNA genotype	10, 11, 12, 13, 27	10	13	12, 27	14, 15, 16, 17	14	29
Max. growth T°C	35–39	38–39	35–36	38–39	33–35	34	37–38
Indole	+	+	+	d	[-]	-	-
DNase	[-]	-	+	-	+	+	-
Urease, Christensen's	d	-	[+]	d	d	[-]	+
Esculin hydrolysis	+	+	+	d	[+]	d	+
Tryptophan deaminase	-	-	d	-	[+]	d	-
Simmons' Citrate	d	+	d	d	d	d	+w
Annular hemolysis (sheep blood agar)	d	+	[-]	+	d	+w	+
Annular hemolysis (horse blood agar)	d	+	-	d	[+]	+	+
Acid production from:							
Mannitol	d	dw	-	+	[-]	-	-
Trehalose	[+] w	+ w	[+] w	[+] w	d	+	[+]
Utilization of:							
L-Fucose	dw	d	-	[+]	d	+	-
DL-Glycerate	-	d	-	-	+	+	d
L(-)Histidine	d	+	[+] w	d	[+]	+	d
myo-Inositol	+	+	+	+	[+]	d	d
DL-Lactate	dw	-	-	d	-	-	-
D-Mannitol	d	+	-	+	[-]	-	-

antibiotics, and other properties usually exhibited by wild clones (named “phase I variants”) freshly isolated from the natural environment. Cells do not reduce nitrate and are proteolytic for gelatin, positive for catalase and negative for oxidase, *O*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG), Voges-Proskauer, arginine dihydrolase, lysine and ornithine decarboxylase. Proteolytic for gelatin. Most strains will hemolyze sheep and/or horse blood, some producing an unusual annular hemolysis on sheep blood at 25°C. All strains are lipolytic on Tween 20, and many are lipolytic on Tweens 40, 60, 80 and/or 85, as well. Cells produce acid from glucose (without gas), fructose, D-mannose, maltose, ribose, and *N*-acetylglucosamine, but only weakly from glycerol. Fumarate, glucosamine, L-glutamate, L-malate, L-proline, succinate and L-tyrosine are utilized as sole carbon and energy sources. Biochemical identification of *Photorhabdus* is summarized in Table 2. The mol% G+C of the DNA is 43–45 (*P. luminescens* strain Bd).

The American Type Culture Collection has the type strain (Hb) under accession number {ATCC 29999}. Unfortunately, it appears that the type strain of both the genus (*Photorhabdus*) and the species (*P. luminescens* strain Hb) is not a good representative of the *Photorhabdus* isolates, in as much as none of the numerous new isolates have been found to belong to this taxon. So far, strain Hm remains the only one closely related to the type strain Hb. The European Molecular Biology Laboratory (EMBL) accession number of the 16S rRNA gene sequence of the strain DSM 3368 (paratype of {ATCC 29999}; Rainey et al., 1995) is X82248.

**PHOTORHABDUS LUMINESCENS** The species characteristics of *Photorhabdus luminescens* (lu.mi.nes'cens. M.L. pres. part. *luminescens*, luminescing; for its luminescence; Thomas and Poinar, 1979; Boemare et al., 1993) are listed in Table 2. Cells are large rods (2–6 by 0.5–1.4  $\mu$ m). There are two phase variants (I and II), both luminous, but phase I has luminescence more than 100-fold greater than phase II. Maximum growth in nutrient broth occurs at 35–39°C. Strains are indole positive. Whereas some strains acidify mannitol, most are weak acid producers from fructose, *N*-acetyl-glucosamine, glucose, glycerol, maltose, mannose, ribose and trehalose. Protein inclusions found in the protoplasm of phase I cells are poorly produced in phase II cells. The natural habitat is in the intestinal lumen of entomopathogenic nematodes of *Heterorhabditis bacteriophora* (Brecon and HP88 subgroups), and of *Heterorhabditis indica*. *Photorhabdus luminescens* is divided in three subspecies. Accession numbers of type strain Hb and of the 16S rRNA gene sequence are given above.

**PHOTORHABDUS LUMINESCENS SUBSP. LUMINESCENS** The subspecies characteristics of *Photorhabdus luminescens* subsp. *luminescens* (Fischer-Le Saux et al., 1999) are listed in Table 2. Maximum growth in nutrient broth occurs at 38–39°C. Cells are positive for esculin hydrolysis, weakly positive for indole, and negative for DNase, tryptophan deaminase and urease. They produce annular hemolysis on sheep and horse blood agars. Cells use mannitol, not DL-lactate, as sole source of carbon and energy. This subspecies is symbiotically associated with nematodes from the Brecon subgroup of *H. bacteriophora*, the type species of the genus *Heterorhabditis* (Poinar, 1976). See accession numbers of type strain Hb and of the 16S rRNA gene sequence above.

**PHOTORHABDUS LUMINESCENS SUBSP. LAUMONDII** The subspecies characteristics of *Photorhabdus luminescens* subsp. *laumondii* (lau.mon'di.i. M.L. gen. n. *laumondii* of Laumond; referring to Dr. C. Laumond, a major contributor to the use of entomopathogenic nematode/bacterial complexes for insect pest control; Fischer-Le Saux et al., 1999) are listed in Table 2. Maximum growth in nutrient broth occurs at 35–36°C. Cells are positive for esculin hydrolysis, indole and DNase, mostly positive for urease and variable for tryptophan deaminase. They show total hemolysis on sheep and horse blood agars (the *Photorhabdus* annular reaction is rare) and do not use L-fucose, DL-glycerate, DL-lactate or mannitol.

Symbiotically, this subspecies (isolated in South and North America, southern Europe and Australia) is associated with nematodes of the HP88 subgroup of *H. bacteriophora*, which is evidenced by the binding of the satellite DNA probe of the nematode strain HP88 (provided by the team of Dr. C. Laumond; Grenier et al., 1996).

Type strain TT01 is held in the Collection of l'Institut Pasteur under accession number CIP 105565. The EMBL accession number of the 16S rRNA gene sequence is {AJ007404}.

**PHOTORHABDUS LUMINESCENS SUBSP. AKHURSTII** The subspecies characteristics of *Photorhabdus luminescens* subsp. *akhurstii* (ak.hurs'ti.i. M.L. gen. n. *akhurstii* of Akhurst; referring to Dr. R. Akhurst, a major contributor to the bacteriological symbionts of entomopathogenic nematodes) are listed in Table 2. Maximum growth in nutrient broth occurs at 38–39°C. Esculin hydrolysis is positive, tryptophan deaminase and DNase negative, urease and indole variable. Annular hemolysis is observed on sheep blood agar, and in some strains, on horse blood

agar. Utilization of DL-lactate as sole source of carbon is variable and weak when positive. Mannitol is used (producing acid) and DL-glycerate not used.

Symbiotically, this subspecies is associated with the nematode *H. indica* isolated in warm regions; the first strain (strain D1) was isolated from Australia (Darwin, Northern Territory) by Dr. R. Akhurst.

Type strain FRG04 is held in the Collection of l'Institut Pasteur under accession number CIP 105564. The EMBL accession number of the 16S rRNA gene sequence is AJ007359.

#### THE SUBSPECIES *PHOTORHABDUS TEMPERATA*

The species characteristics of *Photorhabdus temperata* (tem.pe.ra'ta, L. fem. part. adj. *temperata*, moderate, so named because this species grows at moderate temperature; Fischer-Le Saux et al., 1999) are listed in Table 2. Cells are large rods (2–6 by 0.5–1.4 µm). Two phase variants occur, both of them highly luminous. Maximum growth in nutrient broth occurs at 33–35°C. Strains are positive for DNase, mostly positive for esculin hydrolysis and tryptophan deaminase, mostly negative for indole, and variable for urease. Acid is produced from fructose, *N*-acetylglucosamine, glucose, mannose and ribose; acid production from glycerol and maltose is weak. Protein inclusions are in protoplasm of phase I and II cells, but poorly produced in phase II cells. Annular hemolysis often occurs on sheep and horse blood agars. Most strains use DL-glycerate, and not DL-lactate as sole source of carbon. However, a few strains use mannitol.

The natural habitat is in the intestinal lumen of entomopathogenic nematodes of *Heterorhabditis megidis*, of the NC subgroup of *H. bacteriophora* and of *H. zealandica*.

Type strain XINach is held in the Collection of l'Institut Pasteur under accession number CIP 105563. The EMBL accession number of the 16S rRNA gene sequence is {AJ007405}.

#### *PHOTORHABDUS TEMPERATA* SUBSP. *TEMPERATA*

The subspecies characteristics of *Photorhabdus temperata* subsp. *temperata* (Fischer-Le Saux et al., 1999) are listed in Table 2. Cells are large rods (2–6 by 0.5–1.4 µm). Two phase variants and occasionally several intermediate forms occur, all of which are luminous. Maximum growth in nutrient broth occurs at 34°C. Cells are indole negative, DNase positive, esculin hydrolysis and tryptophan deaminase variable, and mostly urease negative. Annular hemolysis occurs on sheep and horse blood agars in most isolates. Strains use DL-glycerate and L-fucose, and not DL-lactate and mannitol, as the sole source of carbon.

The natural habitat is in the intestinal lumen of entomopathogenic nematodes of the Palaearctic subgroup of *H. megidis*. See above for accession numbers of the type strain XINach and 16 rRNA gene sequence.

*PHOTORHABDUS ASYMBIOTICA* The species characteristics of *Photorhabdus asymbiotica* (a.sym.bio'ti.ca., Gr. pref. *a*, not; M.L. adj. *asymbioticus*, -a, -um, living together; M.L. fem. adj. *asymbiotica*, not symbiotic; Fischer-Le Saux et al., 1999) are listed in Table 2. Cells are rod shaped (2–3 by 0.5–1.0 µm) and produce a yellow or brown pigment. Maximum growth in nutrient broth occurs at 37–38°C. No phase I isolates have been detected, and isolates never adsorb dyes and sometimes weakly produce antibiotics. Cells are negative for lecithinase on egg yolk agar, positive for urease, esculin hydrolysis and Christensen's citrate, but weakly positive for Simmons' citrate. They are negative for tryptophan deaminase, indole and DNase. Acid is produced from fructose, *N*-acetylglucosamine, glucose, maltose, mannose and ribose, but only weakly from glycerol. Protein inclusions are poorly produced. Tween 40 esterase is variable. Annular hemolysis occurs on sheep and horse blood agars. Cells do not use L-fucose, DL-lactate or mannitol.

Natural habitat is uncertain. All isolates are obtained from human clinical specimens. Strain 3265-86 (ATCC 43950) is the designated type strain, as suggested previously by Farmer et al. (1989).

The EMBL accession number of the 16S rRNA gene sequence (Szállás et al., 1997) is {Z76755}.

#### Habitat

In natural conditions, cells of symbiotic *Photorhabdus* are carried in the intestinal lumen of the free-living dauer stage of *Heterorhabditis* (often referred to as the infective juvenile) and insects infected by these nematodes. The bacterial cells are stored and do not multiply in the gut of the dauer host; this is a nonfeeding stage. When infective juveniles infect an insect, they release their symbionts into the body cavity of the insect prey (Fig. 2). Exceptionally, a few *Photorhabdus* strains may be experimentally infective per os (Ragni et al., 1996), some of them possessing entomotoxins acting orally (Bowen et al., 1998b; Guo et al., 1999; see Applications in this Chapter). Nevertheless, natural *Photorhabdus* septicemia without nematode help has not yet been reported from insect sampling. However, it is notable that such collections would be difficult to recover from nature. Whatever the mode of penetration,



when *Photorhabdus* gain entry into the body cavity of the insects, the bacterial cells multiply inducing toxemia and septicemia and the insect dies. Entomopathogenic nematode-infected insect cadavers do not putrefy and the nematode reproduction occurs in a sort of “bag” where the integrity of the cuticle of the insect cadaver seems to be a major barrier to external saprophages. In addition, several antimicrobial barriers acting in the body cavity of the insect contribute by suppressing microbial competitors. *Photorhabdus* produces both antibiotics (see Physiology in this Chapter) possessing a wide spectrum of activity (Akhurst, 1982a; Li et al., 1995; McInerney et al., 1991a; McInerney et al., 1991b; Paul et al., 1981; Richardson et al., 1988; Webster et al., 1998) and bacteriocins acting against closely related species (Baghdiguian et al., 1993; Boemare et al., 1992; Boemare et al., 1997a). These products have been identified from in vitro cultures, and also from in vivo analyses (Hu et al., 1998b), strongly indicating their role in the prevention of bacterial and fungal putrefaction of cadavers. Consequently the carcass is a sort of natural monoxenic microcosm that produces an apparent “mummification.” Nematodes reproduce inside and feed on the insect remains as metabolized by the symbiotic bacteria and also on the bacterial biomass. When the dauers escape the insect cadaver to search for new prey, they carry the symbiont in their gut, ensuring the vertical transmission of the mutualistic association. Although the nematode hosts are the natural vectors of symbiont propagation in the insects, in nutritional terms the *Photorhabdus* associated with nematodes might be considered entomophilic, rather than nematophilic, microorganisms.

However, *P. asymbiotica* is associated with human clinical specimens, not nematodes. These human clinical isolates are the only free-living members so far isolated from the genus. The life cycle of the clinical strains of *Photorhabdus* is much less certain. They have been isolated from five clinical sources in the United States (Farmer

et al., 1989) and five recently in Australia (Peel et al., 1999). Isolations were variously made from tissue, blood and sputum samples. No definite route of infection has been established; two patients reported spider bites prior to the infection and a third reported the possibility of a spider bite, but no definite connection between bite and infection has been made. Although some patients may have been immunocompromised, this was definitely not the case for at least two of the Australian patients. These clinical isolates were all cultivable on standard media at 37°C. Three of the four tested isolates exhibited the annular hemolysis on sheep blood agar at 25°C. By the time they were identified, all were phase II cultures (see Physiology/Phase Variation in this Chapter); it is not known whether they were originally isolated in phase II form.

**COSPECIATION** When the bacterial taxonomic results are compared with the taxonomic data of the host nematodes, a relatedness of the two taxonomic structures is noted, and a phenomenon of cospeciation between bacterium and nematode genera is implied (Table 3). The apparent exceptions do not fundamentally modify the concept because they are essentially the result of taxonomic confusions in the subgroups of *Heterorhabditis bacteriophora*. This species is widely distributed throughout the world, and with the use of more precise methods, nematological taxonomists will probably delineate subspecies. A phenomenon always seems to be in progress: the coevolution between both partners of the symbiosis. How is the specific association between bacteria and nematode maintained? It is likely that signal compounds are involved in the recognition for both partners, and there are indications of a specific attachment by the bacteria to the host cells. When leaving the insect cadaver, dauer larvae reinitiate the symbiosis by the recruitment of symbiont cells, which do not multiply inside the larvae primarily because of the lack of suitable nutrients. So this special association, where bacteria are not digested and are actually very well

Table 3. Species correspondence between *Photorhabdus* and *Heterorhabditis* nematodes.

<i>Heterorhabditis</i>	Symbiont	Secondary guest <sup>1</sup>
<i>H. bacteriophora</i> subgroup Brecon <sup>2</sup>	<i>Photorhabdus luminescens luminescens</i>	
<i>H. bacteriophora</i> subgroup HP88 <sup>3</sup>	<i>Photorhabdus luminescens laumondii</i>	<i>Ochrobactrum</i> spp.
<i>H. indica</i>	<i>Photorhabdus luminescens akhurstii</i>	<i>Ochrobactrum anthropi</i> , <i>O. intermedium</i>
<i>H. zealandica</i>	<i>Photorhabdus temperata</i>	
<i>H. bacteriophora</i> subgroup NC (synonym <i>H. heliothidis</i> )	<i>Photorhabdus temperata</i>	
<i>H. megidis</i> Nearctic group (Ohio, Wisconsin)	<i>Photorhabdus temperata</i>	
<i>H. megidis</i> Palaearctic group	<i>Photorhabdus temperata temperata</i>	<i>Providencia rettgeri</i>
Clinical opportunistic strains (vector candidates: spiders?)	<i>Photorhabdus asymbiotica</i>	

preserved, suggests a special behavior of the dauer larvae, and a special quiescent physiology of the bacteria.

An exciting report of Poinar (1993) suggested that *Heterorhabditis* evolved from a marine ancestor. On the basis of biological, taxonomic and ecological arguments the genus *Heterorhabditis* would come from a *Pellioiditis*-like-ancestor in an arenicolous marine environment. As it seems likely that its symbiotic bacterium would have obtained the *lux* genes by horizontal genetic transfer from marine bacteria, the symbiosis may have originated at the seashore interface. *Photorhabdus luminescens* subsp. *akhurstii* isolated from *H. indica*, restricted to the sandy beaches of the Caribbean basin (Constant et al., 1998), may be a good example. In other parts of the world where complexes of *H. indica* and *P. luminescens* subsp. *akhurstii* also have been isolated, they were found under (or linked to) the sea that in geological times covered the Northern Territory in Australia, Nile Delta in Egypt and Negev Desert in Israel, except in India where they were found inland.

On some rare occasions other bacteria have been isolated from *Heterorhabditis* with *Photorhabdus*, for instance the genus *Ochrobactrum* (Babic et al., 2000) or the genus *Providencia* (Jackson et al., 1995). For all the nematodes sampled and on the basis of 16S RNA genotype and phenotypic properties, the *Ochrobactrum* isolates were correlated with their geographical origin, whereas the corresponding *Photorhabdus* symbiont was only correlated with their host's genotype (Babic et al., 2000). This shows the absence in the former case, and the presence in the latter case, of a cospeciation. Moreover the *Ochrobactrum* (and *Providencia*) probably came from an intercuticular location in contrast to *Photorhabdus*, which comes from the intestine of the host-nematode (see Isolation/Isolation of Other Isolates in this Chapter).

## Isolation

**ISOLATION OF SYMBIOTIC AND NONSYMBIOTIC STRAINS OF PHOTORHABDUS** Three methods have been used for isolating *Photorhabdus* from nematodes. The "hanging drop" uses a sterile drop of insect hemolymph to which surface-disinfected dauer-stage *Heterorhabditis* are added (Poinar and Thomas, 1966b). The nematodes exsheath their old cuticles in the hemolymph drop and commence development, releasing their symbiont, which can be subcultured after about 24 h. A second method is to collect under sterile conditions a drop of insect hemolymph from an insect 24 h after infection by *Heterorhabditis*, and to streak it onto nutrient agar. The third method is to crush about 100 surface-disinfected dauer-

stage *Heterorhabditis* and to streak the macerate onto nutrient agar (Akhurst, 1980). This last method is the most rigorous method for assessing the microflora of the intestine of entomopathogenic nematodes, provided that a suitable control on the effectiveness of the surface disinfection is employed. The three methods have revealed the occurrence of *Photorhabdus* in every *Heterorhabditis* sp.

Human clinical isolates of *P. luminescens* have been variously obtained from open wounds, fluid aspirated from unerupted lumps, blood and sputum. These clinical isolates can be cultured at 37°C.

**ISOLATION OF OTHER BACTERIA FROM HETERORHABDITIS** The presence of secondary bacteria occurring together with the natural symbiont in some samplings of *Heterorhabditis* is noteworthy (see Habitat/Cospeciation in this Chapter). For example, in the case of *H. indica* (Babic et al., 2000), it is easy to discriminate *Ochrobactrum* by simple bacteriological tests (for positive oxidase and nitrate-reductase, for oxidative metabolism) from secondarily developed (see Physiology/Phase Variation in this Chapter) *Photorhabdus* variants (Wouts, 1990), small colony variants (Hu and Webster, 1998a), or intermediate forms of phase variants (Gerritsen et al., 1995). It was believed that these bacteria are carried in the space between the L2 and L3 cuticles and are isolated from infective juveniles when they are not adequately exsheathed during the disinfection of the nematode larvae. The conclusion that these bacteria are occasionally present in the nematodes without any role in the association is quite reasonable (Babic et al., 2000).

## Identification

The taxonomic work necessary to characterize the species requires a long time for collecting the largest possible sample of strains to ensure adequate representation of the biodiversity of the genus and to permit conduct of the polyphasic analyses (see Taxonomy in this Chapter). Bacteriologists have to propose practical methods to identify new isolates more rapidly. This problem is not easy with *Photorhabdus*, which exhibits few positive responses with the classical phenotypic tests used for Enterobacteriaceae. Even if most of the responses are negative, some phenotypic tests must be conducted to confirm the genus identification.

The conventional methods for other Enterobacteriaceae are used to identify the *Photorhabdus* strains, but all biochemical tests are routinely conducted at 28°C and checked after 3–5 days of incubation. Discriminative characters are summarized in Table 2. Light microscopic examina-

tion of the rods at beginning of the stationary period of a culture is particularly notable: there are large, motile (by peritrichous flagella), Gram-negative rods (average 3–5 by 1–1.5  $\mu\text{m}$ ), sometimes up to 10  $\mu\text{m}$  long, containing highly diffractive protoplasmic inclusions.

*Photorhabdus* strains are easily distinguished phenotypically from *Xenorhabdus* spp. because luminescence and catalase, both physiologically very significant characters, are positive for *Photorhabdus* and negative for *Xenorhabdus*. Urease in most *Photorhabdus* strains is positive, and assimilation of DL-lactate is negative for *Photorhabdus*; annular hemolysis on sheep blood agar at 25°C is only observed with *Photorhabdus* strains.

Luminescence does not cause confusion with the light-emitting marine bacteria of other families. *Photorhabdus* is differentiated from *Vibrio*, *Alteromonas* and *Photobacterium* by having peritrichous, nonpolar, flagella and in not requiring sodium ions for growth. Luminescence for most phase I variants (see Physiology/Phase Variation in this Chapter) can be checked in a darkroom after 10 min for dark-adaptation of eyes. To assess absence or weakness of light production in the phase II variants, a scintillation counter, a fluorimeter or a photomultiplier must be used. A loopful of an agar culture of each phase-variant culture is suspended in 10 ml of distilled water in a scintillation vial for immediate counting with a fully opened window setting (Grimont et al., 1984).

Another important phenomenon is the phase variation that essentially appears during in vitro culture (see Physiology/Phase Variation in this Chapter). Details of the techniques for identifying the phase variants have been summarized recently (Boemare et al., 1997b). The essential points are the following. Adsorption of dyes as described by Akhurst (1980) is the most convenient test to characterize the phase variants. MacConkey agar, or better still MacConkey agar without the bile salts, is a good medium for distinguishing phase I (red colonies) and phase II (off-white or yellow) variants (Boemare and Akhurst, 1988). On the nutrient bromothymol blue agar (NBTA) NBTA medium described by Akhurst (1980), the adsorption of bromothymol blue by *Photorhabdus* may be confused by the pigmentation of strains, and the resulting color of the clones can be difficult to distinguish. As most *Photorhabdus* are pigmented, growth on nutrient agar is often sufficient to differentiate clones of the variants, which differ significantly in not only pigmentation but also colony morphology, with phase I mucoid and convex and phase II non-mucoid and flattened. To test antibiotic production by *Photorhabdus*, clones of both variants are spot-inoculated on nutrient agar plates. After growth (generally 48 h later),

cultures are killed by chloroform vapor (30 min) and covered by fresh nutrient semisolid agar (0.6%) inoculated with a bacterial indicator such as *Micrococcus luteus* (Akhurst, 1982a). The inhibition halos of the indicator culture denote the phase I variants.

Antibiograms (Bauer et al., 1966) must be done at 28°C and incubated for 3 days to observe clear zones. *Photorhabdus* have large zones of inhibition around disks impregnated with nalidixic acid, gentamycin, streptomycin, kanamycin, tetracycline, and chloramphenicol, but none around penicillin. Resistance to colistin, ampicillin, carbenicillin and cephalothin is variable from strain to strain (Farmer, 1984). It is interesting to note that one patient infected with an isolate sensitive to gentamycin in vitro did not respond to gentamycin treatment (M. Peel, personal communication). The major cellular fatty acids of *Photorhabdus* are C<sub>16:0</sub> and C<sub>18:1</sub>, with C<sub>i-15</sub>, C<sub>i-17</sub> and C<sub>16:1</sub> being major components in some strains (Janse and Smits, 1990; Suzuki et al., 1990). Ubiquinone-8 is the respiratory quinone in all strains (Suzuki et al., 1990).

In addition to phenotypic tests, some other simple positive and reliable tests are needed to identify *Photorhabdus* strains. These last four years, molecular probes useful for *Photorhabdus* ribotyping have identified defining restriction patterns specific for each group by using PCR-RFLP of the 16S rRNA genes. Pütz et al. (1990) initiated the use of the variable region of *Photorhabdus* and *Xenorhabdus* 16S rRNA genes to prepare oligonucleotide probes for the ribotyping of both genera, and Ehlers and Niemann (1998a) proposed to extend more precisely this method for *Photorhabdus*. Probes were prepared by PCR and amplified from a primer chosen in the variable region at positions 450–480 (*E. coli* numbering) and a primer from a region highly conserved at the positions 795–755 according to Stackebrandt and Goodfellow (1991). Thus, Ehlers and Niemann (1998a) defined a “type” probe for identifying the type species of the genus, a “tropicus” probe for symbionts from tropical nematodes, and a “temperatus” probe for the symbionts of nematodes from temperate climates. Today more than 22 total and 28 partial 16S-rDNA sequences are available on the gene databases covering most of the diversity of the *Photorhabdus*, allowing one to check the specificity of such variable regions for each species or subspecies or both. Some of the results (Table 4) are in accordance with the definition of a subspecies, with sequences belonging to a given subgroup showing the same variable region. This is the case for the “temperatus” probe, which has a specific sequence identical to sequences of several of the Palaearctic strains and to the sequence of *Pho-*



rRNA genes for identifying the diversity of the *Photorhabdus* and *Xenorhabdus*. (See *Photorhabdus*/Taxonomy and *Xenorhabdus*/Taxonomy in this Chapter) Fischer-Le Saux et al. (1998) applied this PCR-RFLP method (Fig. 1). Based on the total amplified sequence of the 16S ribosomal genes, the method provides reference restriction patterns for identifying other isolates. The reliability of results obtained with these genotypes and those from the previously described probes (Ehlers and Niemann, 1998) is at present uncertain. The latter could be improved by the choice of more specific variable sequences covering all the *Photorhabdus* biodiversity. Consequently, both molecular probes for and PCR-RFLP analysis of the 16S rRNA genes promise to be efficient tools for rapid identification of *Photorhabdus* isolates.

### Cultivation

In vitro subcultures from fresh isolates can be obtained without major difficulty. *Photorhabdus* are mesophilic bacteria able to grow between 15 and 35°C, and some strains at 37–38°C. Subculturing and all biochemical tests should be undertaken around the optimal temperature of 28°C. Usually, nutrient agar or Luria-Bertani agar are sufficient for growth. On minimal media, nicotinic acid, *para*-aminobenzoic acid, proline, tyrosine and serine are required as growth factors, the mix of growth factors varying between strains (Grimont et al., 1984). Minimal medium II (BioMérieux®) contains all the necessary requirements to test utilization of organic compounds.

### Preservation

The standard methods of freeze-drying, or low temperature storage (liquid nitrogen or at –80°C) used for Enterobacteriaceae, are also useful for long-term storage of *Photorhabdus* strains; –20°C is unsatisfactory. Cultures do not survive more than few months in broth or on agar plates at room temperature, and phase variation (see Physiology/Phase Variation in this Chapter) is likely to occur in this time. Cultures can be routinely maintained for one month at 15°C, but storage at 4°C is unsuitable; to prevent phase variation, phase I clones have to be subcultured every week from the neutral red dye-adsorbing clones on MacConkey agar.

### Physiology

**PHASE VARIATION** In *Photorhabdus*, the phase variants differ by many characters and this is demonstrated by using a multivariate correspondence analysis of the phenotypic data (Fig. 7). All

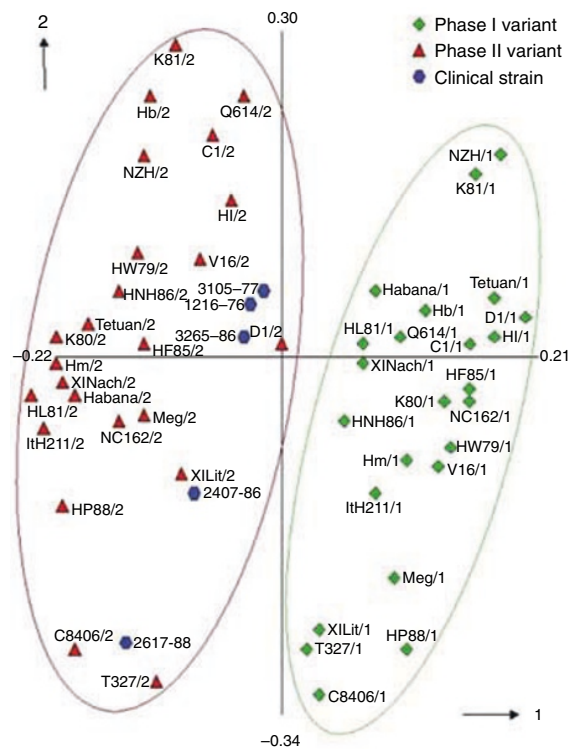


Fig. 7. Multivariate correspondence analysis of phenotypic data from *Photorhabdus* phase variants. All the data published by Akhurst and Boemare (1988) were treated as two-state (0,1) characters. Characters that were positive or negative for all isolates (i.e., that were not discriminative) were deleted (180/240). Here, the phase variants are positioned in the A space defined by the two first components using Q-R reciprocal analyses of the GENSTAT software (Alvey et al., 1980). Correspondence analysis from the contingency table used in Akhurst et al. (1996).

phase I variants are aggregated on the right of the figure, whereas phase II variants are on the left. Note that clinical strains are included in the cluster of phase II variants. No clinical isolates that resemble phase I nematode symbionts have ever been reported. When phase characters are excluded from the numerical analysis, the taxonomic clusters corresponding to the species are very well defined, meaning that phase variation is an infraspecific common phenomenon that does not essentially affect the taxonomy (Fig. 6).

*Photorhabdus* phase I variants are differently pigmented from phase II variants (e.g., red in phase I and yellow in phase II). Larger than phase II cells, phase I cells also are pleomorphic, comprising rods (80–90%) and spheroplasts (10–20%), and harbor protoplasmic inclusions (Boemare et al., 1983c; Fig. 8). Although the shift from phase I to II is spontaneous, it is remarkable that the wild dauer *Heterorhabditis* almost exclusively harbor phase I *Photorhabdus* (Akhurst and Boemare, 1990). The role of phase

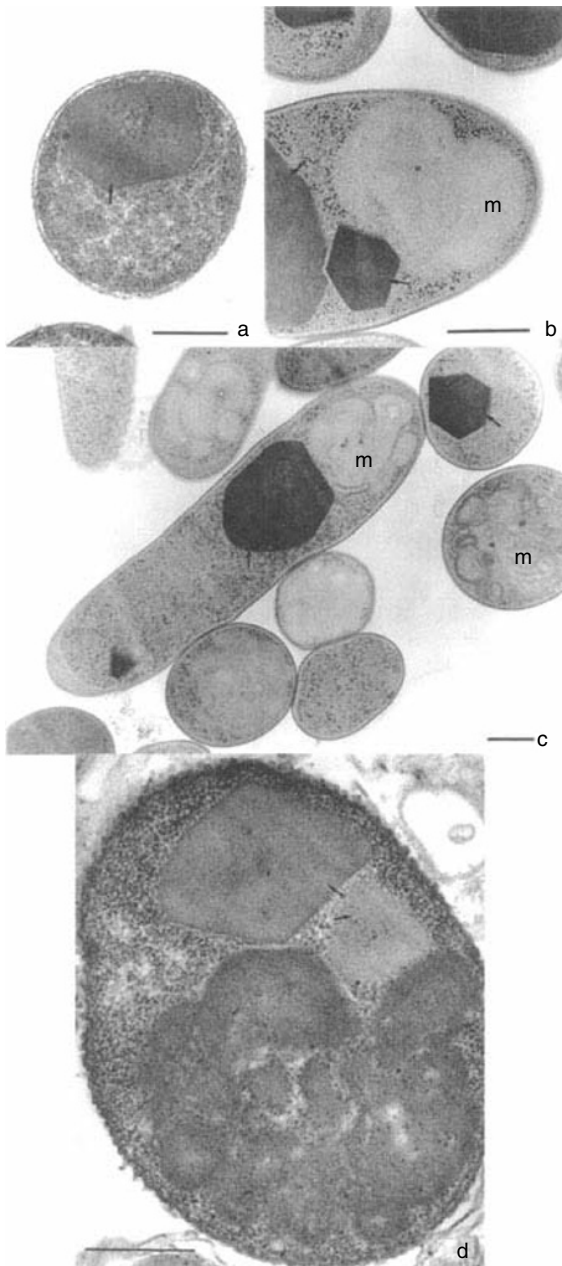


Fig. 8. Protoplasmic inclusions in *Photorhabdus*. (a) Transmission electron micrograph of *Photorhabdus temperata* strain NC1. Culture is in stationary growth on nutrient agar, fixed with 2.5% glutaraldehyde + 1% osmium tetroxide, and stained with uranyl acetate and lead citrate. Arrow = crystalline inclusion. Bar = 0.5  $\mu\text{m}$ . (b) Fast-freeze fixation and cryosubstitution of *Photorhabdus temperata* strain NC1. Culture is in stationary growth on nutrient agar. Piece of solid agar bearing bacterial colonies was freeze fixed by liquid helium at  $-260^{\circ}\text{C}$  on pure copper, cooled, dehydrated by 100% ethanol, and substituted by infiltration with a cryocool for 3 days at  $-90^{\circ}\text{C}$ . Sections were contrasted with uranyl acetate and lead citrate. Two types of crystalline inclusions (arrows) are shown. The corresponding structures similar to “myelinic” (m) membranes shown with strain Q614 (Fig. 8d) are poorly contrasted with this method (see Baghdiguian et al., 1993). Bar = 0.5  $\mu\text{m}$ . (c) Fast-freeze fixation and cryosubstitution of *Photorhabdus temperata* strain NC1. Culture is in stationary growth on nutrient agar. Specimen preparation was by the method described in legend to Fig. 6b. Membranes (m) of “myelinic” bodies are better visualized than they are in Fig. 8b (see Baghdiguian et al., 1993). Arrows = crystalline inclusions. Bar = 0.5  $\mu\text{m}$ . (d) Cell of nonluminous strain Q614 of *Photorhabdus* sp., fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, and stained with uranyl acetate and lead citrate. Two types of crystalline inclusions (arrows) are shown in the protoplasm. Significance of the condensed material looking like fingerprints and similar to “myelinic” bodies visible in the two thirds of the protoplasm is not interpreted as yet. Bar = 0.5  $\mu\text{m}$ .

II variants is not evident and today the data to support a good explanation for their occurrence is unconvincing.

Phase change occurs during the in vitro stationary period of growth in a highly unpredictable manner (Akhurst and Boemare, 1990). The two phases of *Photorhabdus* differ significantly in respiratory activity (Smigielski et al., 1994b). After periods of starvation, phase II cells recommenced growth within 2–4 h of the addition of nutrients, compared with 14 h for phase I cells, indicating the former had more efficient nutrient uptake ability. Phase II variants may grow a little on complex media previously utilized by phase I variants (Akhurst and Boemare, 1990). How-

ever, although reciprocal phase change occurs in *Xenorhabdus*, conversion from phase II *Photorhabdus* to phase I has not been unambiguously demonstrated. Several intermediate colony forms, possessing at least some phase I properties, have been recorded (Gerritsen et al., 1992). However, it is not certain that these colonies were not mixtures of phase I and II cells, rather than truly intermediate. The only confirmed reversion to phase I occurred from intermediate variants not yet established as definitively phase II (Krasomil-Osterfeld, 1995). *Photorhabdus* phase II variants support the growth of their host nematodes very poorly. Consequently the phase traits of *Photorhabdus* are mainly involved in the growth and development of the *Heterorhabditis* nematodes and may be considered to be “symbiotic characters.” However, both phases show a similar entomopathogenic effect and share all the other bacteriological properties of members of the genus.

**PRODUCTION OF SECONDARY METABOLITES** *Photorhabdus* phase I variants produce a variety of secondary metabolites, some of which have antimicrobial properties. The carcass is a sort of monoxenic microcosm where the symbionts eliminate competitive microorganisms by using several antibiotics (see Habitat in this Chapter) possessing a wide spectrum of activity (Li et al.,

1995; Paul et al., 1981; Richardson et al., 1988). Two chemical groups have been characterized: hydroxystilbenes and polyketides (anthraquinone derivatives; Table 5). Hu et al. (Hu et al., 1997; Hu et al., 1998b) purified these compounds and examined the conditions under which these secondary metabolites are produced both in vivo and in vitro culture using a strain of *P. temperata* that was a symbiont of *H. megidis*. Only one of the two identified stilbenes produced in the extract from nematode-bacterium infected insects is recovered from tryptic soy broth culture. An array of closely related polyketide derivatives is also produced by *Photorhabdus* in the insect larvae of *Galleria mellonella*. These pigments vary in the positioning and/or number of hydroxyl and methoxyl groups around the central anthraquinone ring structure (Hu et al., 1998b), and those that are antimicrobial are mentioned in Table 5. Their occurrence also varies according to in vivo or in vitro production. The available nutrients and the prevailing environmental conditions, undoubtedly have a significant effect on the difference in metabolite composition (Webster et al., 1998). These

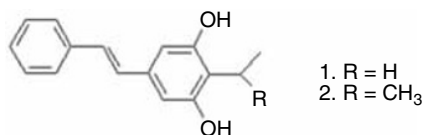
antibiotics are most commonly active against Gram-positive bacteria, but some are effective against Gram-negative bacteria (see Applications in this Chapter).

*Photorhabdus* strains are the only terrestrial luminous bacteria known today. The chemical pathway for producing light is similar to the mechanism of marine bacteria (Frackman et al., 1990b; Frackman and Nealson, 1990a). The luciferase catalyzes the reaction in which reduced flavin mononucleotide (FMNH<sub>2</sub>) and an aldehyde are oxidized to FMN, an acid and H<sub>2</sub>O, and light is emitted at 490 nm. Conversely, the fatty acid reductase complex generates an aldehyde and the flavin oxidoreductase reduces FMNH<sub>2</sub>. No satisfactory demonstration has yet been provided to explain the role of luminescence in this genus. An association between luminescence and virulence factors for the pathogenicity of *Vibrio harveyi* against *Penaeus monodon* has been noted (Manefield et al., 2000).

**BACTERIOCIINOGENY** Unlike *Xenorhabdus* spp., no temperate phages have been yet characterized in *Photorhabdus* strains, although phage

Table 5. Secondary antibiotic metabolites from *Photorhabdus* strains.

1. *Trans*-stilbenes



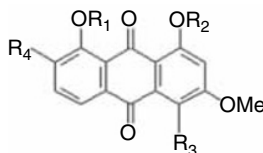
R substituents	In vivo production	In vitro production	pigmented	active against
3,5-dihydroxy-4-ethyistibene	+	–	–	bacteria, fungi
3,5-dihydroxy-4-isopropyistibene	+	+	+	bacteria, fungi, nematodes

Species: *P. luminescens luminescens* Hb, Hm

*P. temperate* Nearctic group NC19, C9, HK

(Hu et al., 1998; Li et al., 1995; Paul et al., 1981; Richardson et al., 1988; Sundar and Chang, 1992)

2. Polyketides



R1, R2, R3 substituents	<i>in vivo</i> production	<i>in vitro</i> production	pigmented	active against
3,8-dimethoxy-1-hydroxy-9,10-anthraquinone	+	+	yellow	bacteria
1,3-dimethoxy-8-hydroxy-9,10-anthraquinone	+	+	yellow	bacteria
1,8-dihydroxy-3-methoxy-9,10-anthraquinone	+	–	yellow	bacteria
1-hydroxy-2,6,8-trimethoxy-9,10-anthraquinone	+	–	yellow	bacteria
1,4-dihydroxy-2,5-dimethoxy-9,10-anthraquinone	+	–	red	bacteria

Species: *P. luminescens luminescens* Hb, Hm

*P. temperate* Nearctic group NC19, C9, HK

(Hu et al., 1998; Li et al., 1995; Paul et al., 1981; Richardson et al., 1988; Sztaricskai et al., 1992)

tail-like bacteriocins have been observed by electron microscopy of culture supernatants (Baghdigui et al., 1993). They are different in size and shape than those of *Xenorhabdus* (Boemare et al., 1992). It is reasonable to think that they play also a role in the competition with closely related bacterial genera, as demonstrated for *Xenorhabdus* (Boemare et al., 1992; Thaler et al., 1995; Thaler et al., 1996; Thaler et al., 1997).

## Genetics

Genetic studies, undertaken over these last ten years, examined several structural genes and attempted to identify the mechanism of phase variation (for a comprehensive review see: Forst et al., 1997; Forst and Neilson, 1996). Recently the characterization of genes encoding protein toxins that (when ingested) are active against insects was described (Blackburn et al., 1998; Bowen et al., 1998b), and two corresponding toxic proteins have been purified (Guo et al., 1999).

Several strains of *Photorhabdus* harbor megaplasmids for which no role or gene has been identified (Smigielski and Akhurst, 1994a). Megaplasmids in other species are known to contain genetic information necessary for symbiosis, pathogenicity and conjugation. *Photorhabdus* plasmids have not been further characterized or engineered to generate cloning vectors.

**GENES AND PROTEINS** Several genes have been identified in *Photorhabdus* strains (Table 6). The *lux* operon (*luxCDABE*), which encodes the proteins required for luminescence (Frackman et al., 1990b) has been characterized. The amino acid identity values for the corresponding proteins coded by the structural genes of the marine luminous bacteria *Photobacterium* and *Vibrio* are 62–85% for *lux A* and 47–59% for *lux B* (Johnston et al., 1990; Meighen and Szittner, 1992). The regulatory genes for the *Photorhabdus lux* operon have not yet been identified (Szittner and Meighen, 1990; Xi et al., 1991).

The operon coding for polynucleotide phosphorylase (Pnp), which degrades mRNA, and for

a ribosomal protein, RpsO, was cloned from the strain K122 (Clarke and Dowds, 1994). Regulation of expression of the *rpsO-pnp* operon is complex. The two genes are induced at low temperature.

A *malB* region with 53.5% identity to the equivalent *E. coli* gene was identified (Dowds, 1997). The maltose regulon of *E. coli* consists of genes involved in maltose uptake. The cloned region encompasses part of the *malE* and *malK* genes and the regulatory region between them. The intergenic region contains the control regions that respond to levels of maltose in the media and also to catabolite repression.

Phase I variants produce a pigment whose color varies according to the strain. The genes coding for the red anthraquinone pigment of the Hm strain have been cloned into *E. coli* (Frackman and Neilson, 1990a).

The genes *cipA* and *cipB* coding for the two types of protein crystals from the protoplasm of *Photorhabdus* (Boemare et al., 1983c; Couche et al., 1987b; Couche and Gregson, 1987a), have been characterized (Bintrim and Ensign, 1998). These genes have separate promoters and when expressed in *E. coli* resulted in inclusion body formation in the cells. Normally these genes are strongly expressed only by phase I variants (Boemare and Akhurst, 1988), so their inactivation induces changes in some other phase-related characters.

The *lip-1* gene of extracellular lipase was also cloned and sequenced (Wang and Dowds, 1993). It encodes a protein of 645 amino acids from which a hydrophobic leader sequence of 24 amino acids is removed during the processing in *E. coli*. Such a processing seems to be necessary for the secretion of other active proteins.

Toxin genes have been cloned from strain W14 (Bowen et al., 1998b). These genes encode large insecticidal toxin complexes with little homology to other known toxins. The toxin purified as a protein complex that has an estimated molecular weight of 1,000,000. It consists of a series of four native complexes at loci named *tca*, *tcb*, *tcc*, and *tcd*. Both *tca* and *tcd* encode complexes with high

Table 6. Identified genes and proteins from *Photorhabdus* strains.

Gene	Protein encoded	% identity	with
<i>luxA</i> (4)	luciferase alpha-subunit	85	<i>Vibrio harveyi</i>
<i>luxB</i> (4)	luciferase beta-subunit	60	<i>Vibrio harveyi</i>
<i>cipA</i> , <i>cipB</i> (1)	protoplasmic inclusions	none	
<i>pnp</i> (3)	polynucleotide phosphorylase	86	<i>Escherichia coli</i>
<i>rpsO</i> (3)	ribosomal protein S15	86	<i>E. coli</i>
<i>lip-1</i> (5)	Tween 80 lipase	none	
<i>tca</i> , <i>tcb</i> , <i>tcc</i> , <i>tcd</i> (2)	toxin complexes for insects	not reported	several loci in <i>Yersina pestis</i> genome

**References:** (1) Bintrim and Ensign, 1998; (2) Bowen et al., 1998; (3) Clarke and Dowds, 1994; (4) Szittner and Meighen, 1990; (5) Wang and Dowds, 1993



oral toxicity for insects. Two purified native toxins A and B were found to be active in nanogram concentrations against insects. Two peptides present in toxin B could be processed *in vitro* from a 281-kDa protoxin by endogenous *P. luminescens* proteases (Guo et al., 1999). A smaller insecticidal protein (ca. 40 kDa) unrelated to the large toxin complex has also been identified in *Photorhabdus* and the cognate gene cloned (East et al., 1999).

The sequencing of the whole genome of strain TT01, which is in progress, will identify new genes, see the (<http://www.pasteur.fr/externe>{Institut Pasteur}) for further functional genomic studies.

**REGULATION OF PHASE VARIATION** Major changes in DNA structure do not appear to account for the phenotypic switches. No differences in plasmid profiles of the two phases have been detected. DNA-relatedness studies and restriction digests of total DNA indicated that the organization of the genome is the same in the two phases (Akhurst et al., 1992; Boemare et al., 1993; Frackman et al., 1990b; Wang and Dowds, 1991; Wang and Dowds, 1993).

DNA has been successfully transferred by means of transformation and conjugation. Modification systems such as methylation are commonly associated with restriction endonucleases that protect the genome from foreign DNA. Endonuclease activities have been detected in a range of *Photorhabdus* spp. and an isoschizomer of XhoI was purified from strain K80 of *P. luminescens* subsp. *laumondii* (Akhurst et al., 1992). Such a restriction-modification system is an obstacle for transformation and conjugation of *Photorhabdus* strains and explains the low frequency of transformants and transconjugants obtained. Nevertheless, Frackman and Nealson (1990a) reported the transformation of strain Hm by using competent cells from a modified CaCl<sub>2</sub>/RbCl method, and Dowds (1997) used electroporation to transform with pBR322 and pHK17. Conjugation from *E. coli* to *Photorhabdus* strain K122 allowed transfer of the plasmids RP4 and pSUP104 (Dowds, 1997).

The molecular mechanism of phase variation remains uncertain. Some experiments to inactivate genes involved in phase variation have been successful and indicated that phenotypic phase characters can be altered in phase I variants or expressed in phase II variants. For instance inactivation of the crystal protein genes of strain NC1 produced a phase II phenotype (Bintrim and Ensign, 1998). The UV mutagenesis of phase II variants of strain K122 induced expression of phenotypic properties of the corresponding phase I variants (Wang and Dowds, 1993). The lipase gene transcription is initiated at the same

site in the two phases (Wang and Dowds, 1993) and lipase mRNAs accumulate to the same extent in the two phases, implying that gene expression in the phase II variant is repressed at a post-transcriptional level. In fact the lipase protein accumulates and is secreted to the same extent in the two phases. However the lipase protein is inactive in the phase II variant, as is the protease (Wang and Dowds, 1993). Lipase and protease are regulated at a post-translational level in the Irish K122 strain of *P. luminescens* (Wang and Dowds, 1993), whereas the *lux* genes are post-transcriptionally regulated in the Hm strain (Hosseini and Nealson, 1995). This contrasts with *X. nematophilus* in which the flagellin genes are not transcribed during phase II (Givaudan et al., 1996). These preliminary studies seem to indicate that phase-specific genes in *Photorhabdus* are regulated at a post-transcriptional, probably post-translational, level by a common control mechanism, a genetic level different from that in *Xenorhabdus*.

## Ecology

Despite the intestinal location of *Photorhabdus*, which allows environmental contamination, their specificity for *Heterorhabditis* is a remarkable feature of this symbiosis (Boemare et al., 1997a; Forst et al., 1997). When microbial ecology studies are undertaken, using a simple and fast method of PCR-RFLP of 16S RNA (Brunel et al., 1997), a clear correspondence between *Photorhabdus* isolates and nematode species can be seen. From a total of 75 isolates identified in the Caribbean region (Fischer-Le Saux et al., 1998), two genotypes were associated only with *Heterorhabditis bacteriophora* and another two only with *Heterorhabditis indica*, although these four genotypes belong to the same DNA/DNA relatedness group.

Within the *Photorhabdus* genus, the maximum growth temperature appears to be a relevant taxonomic character defining a critical value for each subgroup (Table 2). It implies essential physiological adaptations in the enzymatic machinery of the bacteria and must therefore be considered as important for defining taxa. Moreover, if we examine the ecology of the corresponding strains, it is notable that the maximum growth temperature and the host species origin are correlated. Symbionts growing at temperatures up to 35–39°C are harbored by *H. bacteriophora* (Brecon, HP88 groups) and *H. indica* that occur in hot regions. Those that grow only up to 35°C are harbored by *H. megidis* (both its Palearctic and Nearctic strains) and *H. bacteriophora* (NC group); all of these nematodes live in temperate climates. Thus, temperature tolerance appears to be an important property reflecting

a long-term adaptation to different climatic conditions.

Two ecological niches have been identified for nematode-symbiotic *Photorhabdus*: one is in the insect host as a metabolically active form and the other is in the gut of the nonfeeding dauer nematode as a quiescent form. The occurrence of nonsymbiotic clinical strains possessing the phase II properties of the nematophilic symbionts, and the remarkable differences in respiratory activity between the two phases of the symbiotic strains, suggest that the soil may be a third niche. However, recently reported experiments to test the symbiotic strains' ability to grow and survive in external environments indicate that they disappear within a few days (Morgan et al., 1997). *Photorhabdus* strains may enter into a nonculturable but viable survival strategy, as do *Aeromonas*, *Vibrio*, *E. coli* and *Salmonella* spp.

### Epidemiology

All *Photorhabdus* strains examined to date have been reported to be entomopathogenic, the LD<sub>50</sub> usually being <100 cells when injected into hemocoel of the insect *Galleria mellonella* (Akhurst and Boemare, 1990; Akhurst and Dunphy, 1993; Farmer et al., 1989). *Photorhabdus asymbiotica* may also cause some human diseases (see Disease).

To test insect pathogenicity, 10<sup>2</sup>, 10<sup>3</sup> or 10<sup>4</sup> cells (total count) from a 24-h broth culture are injected into final instar *Galleria mellonella* or *Spodoptera littoralis* larvae (Lepidoptera). The injected larvae should be placed on dry filter paper in Petri dishes and incubated at 25°C for 3 d. Most *Photorhabdus* spp. will kill <50% at a dosage of 10<sup>2</sup> cells; all will kill <50% at 10<sup>4</sup> cells. Depending on the insect species tested, some differences in pathogenicity between *Photorhabdus* strains and phases may be noted. According to Bucher (1960), when the LD<sub>50</sub> is >10<sup>4</sup> cells, the bacterium may be considered as entomopathogenic. To date, all *Photorhabdus* isolates, regardless of phase, have been reported to infect insects at a LD<sub>50</sub> > 10<sup>2</sup>–10<sup>3</sup> cells by injection. Consequently *Photorhabdus* have to be considered as highly entomopathogenic. In addition some of them (strain W14, and an unnamed Italian strain) may be entomopathogenic by ingestion (French-Constant and Bowen, 1999; Ragni et al., 1996). The toxin produced is active on the insect digestive epithelium, not only from the gut lumen, but also from the body cavity (Blackburn et al., 1998; see Applications in this Chapter).

### Disease

*Photorhabdus* has also been isolated from human wounds and blood in the United States

(Farmer et al., 1989) and more recently in Australia (Peel et al., 1999). None of the infections were lethal but some required weeks of treatment. Four American patients were elderly people or apparently immunocompromised; the other was a 45-year-old male who had suffered a spider bite. The bacterial strains, isolated from them and kept in the United States Centers for Disease Control and Prevention in Atlanta (CDC), were compared after the description of the symbiotic strains was published, and they were defined as a clinical group in the genus (Farmer et al., 1989), and now belong to *P. asymbiotica* (see Taxonomy in this Chapter). Among the four recent isolations in Australia, one of the Australian patients was definitely a victim of spider bite, and another may have been. None of the patients was immunocompromised; the one elderly patient made the fastest recovery. One case was the result of a disseminated infection, whereas the three others had a bacteremia or skin lesions (Peel et al., 1999). The possibility that spiders may vector infection by nonsymbiotic strains of *Photorhabdus* has yet to be tested.

### Applications

Knowledge about the nutritional requirements provided by the symbionts would improve the mass production of the entomopathogenic nematodes for biological control of insect pests. In terms of symbiosis, this is one of the most exciting subjects related to these bacteria. During the industrial process, the quality of the symbiont inoculum, in terms of viability, phase I variant selection, and preservation from any other microbial contamination, must be maintained (Ehlers et al., 1998b; Ehlers et al., 1990). Several programs have been undertaken to define the key factors of the symbiosis; nothing significant has been reported to date. However it should be noted that academic access to this knowledge is difficult to obtain because the know-how of such industrial processes is kept secret. What biochemical compound(s) is/are released in the supernatants in the bioreactors during monoxenic nematode production? A "food signal," a term taken from *Caenorhabditis elegans* physiology, is produced by a German strain of *P. temperata*. Although not produced at the same strength as found in insects, the food signal has been characterized (Strauch and Ehlers, 1998). It induces the exit from the developmentally arrested dauer larvae, explaining the recovery of juveniles when they enter the insects. The food signal could be a special nutrient supplied by the bacteria to the nematode and/or a hormone controlling nematode development.

Some of the secondary metabolites have commercial potential in the pharmaceutical and agro-forestry industries (Webster et al., 1998). Among the isolates examined to date from *P. luminescens* subsp. *luminescens* and *P. temperata*, some of the anthraquinone pigments and the *trans*-stilbenes are antibacterial and the *trans*-stilbenes are antifungal, especially when the isolates are cultivated *in vitro*. This is of great interest to industry (Table 5).

Moreover, the occurrence of protein toxins produced by some *Photorhabdus* strains and acting orally against several insect species has recently enhanced the interest in these bacteria for biological control (Blackburn et al., 1998; Bowen et al., 1998b; Bowen and Ensign, 1998a; Guo et al., 1999). The toxin genes can be cloned and will be inserted into plant genomes, as with *B. thuringiensis* (“Bt”), used for producing transgenic plants toxic for insects. In addition, to overtake the first resistance observed in the target insects consuming these transgenic Bt plants, the insertion of both (Bt and *Photorhabdus*) toxin genes in the same plant can be envisioned (French-Constant and Bowen, 1999). This second field of industrial projects opens very promising biotechnology programs for the next ten years in plant protection. To date, six patents have been submitted (East et al., 1999; Ensign et al., 1997; Ensign et al., 1998; Kramer et al., 1999b; Ragni et al., 1996).

## Genus *Xenorhabdus*

### Phylogeny

Analyses of 16S rDNA sequences show that *Xenorhabdus* is most closely related to *Photorhabdus*. The next nearest phylogenetic neighbors are *Proteus vulgaris* and *Arsenophonus nasoniae* (Brunel et al., 1997; Fischer-Le Saux et al., 1999b; Liu et al., 1997; Suzuki et al., 1996; Szállás et al., 1997). Only limited 16S rDNA sequence data are available at this time, most *Xenorhabdus* species being represented by sequences from only one or two strains. However, *Xenorhabdus* can be distinguished from its nearest phylogenetic neighbor, *Photorhabdus*, by the sequence TTCG at positions 208–211 (*E. coli* numbering) of the 16S rDNA. *Photorhabdus* has a longer version (TGAAAG; Fischer-Le Saux et al., 1999b; Szállás et al., 1997). The PCR-RFLP analysis shows that of the 17 *Xenorhabdus* genotypes identified to date, 8 correspond to 5 species of *Xenorhabdus* and the remainder corresponds to some other, as yet undefined, species (Fig. 9).

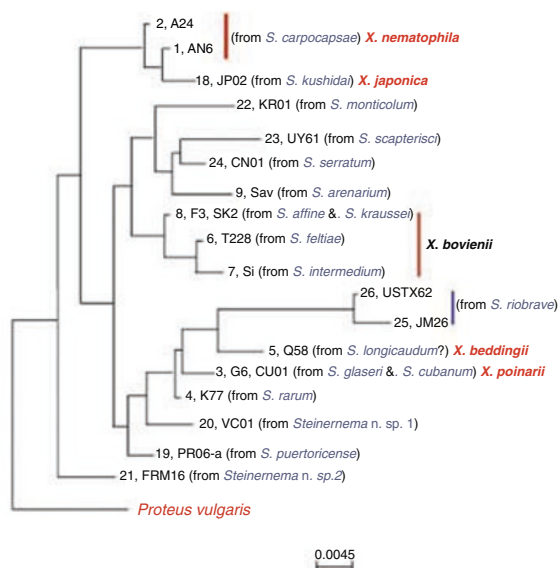


Fig. 9. Phylogenetic tree from PCR-RFLP 16S rDNA genotypes of *Xenorhabdus*. The neighbor-joining method was applied to the 17 defined genotypes, from a total of 65 strains. The number of the genotype according to Fischer-Le Saux et al. (1998) is followed by the name of the representative strain. Defined symbiont species are indicated in red, and host nematodes in blue.

### Taxonomy

**NONMENCLATURE AND FAMILY LINKING** The first symbiotic bacterium isolated from entomopathogenic nematodes was described as a new species “*Achromobacter nematophilus*” (Poinar and Thomas, 1965). With the rejection of the genus “*Achromobacter*” (Hendrie et al., 1974), this new species could not be accommodated within any existing genus. Thomas and Poinar (1979) described a new genus, *Xenorhabdus*, to accommodate the bacterial symbionts of entomopathogenic nematodes as two species, *X. nematophilus*, symbionts of the family Steinernematidae, and “*X. luminescens*,” associated with the Heterorhabditidae; the latter was subsequently reassigned to the genus *Photorhabdus* (Boemare et al., 1993). *Xenorhabdus* have low DNA/DNA relatedness (4%) to the type species of the type genus in the family Enterobacteriaceae (Farmer, 1984) and lack nitrate reductase, which is positive for all other genera in this family. However, they do have the enterobacterial common antigen (Ramia et al., 1982) and phylogenetic analyses based on 16S rDNA (Brunel et al., 1997; Fischer-Le Saux et al., 1999b; Liu et al., 1997; Suzuki et al., 1996; Szállás et al., 1997) confirm their relatedness to the Enterobacteriaceae.

**THE POLYPHASIC APPROACH TO GENUS AND SPECIES DELINEATION** From a phenotypic study of bacterial symbionts of the Steinernematidae,

four groups were recognized within the genus, and the subdivision of *X. nematophila* into subspecies was proposed (Akhurst, 1983). A more comprehensive phenotypic study (Boemare and Akhurst, 1988) led to the elevation of the subspecies to species status, as *X. nematophila* (= *nematophilus*), *X. bovienii*, *X. poinarii* and *X. beddingii* (Akhurst and Boemare, 1988). *Xenorhabdus japonica* (= *japonicus*), symbiotically associated with *Steinernema kushidai*, was described later (Nishimura et al., 1994). DNA/DNA hybridization (Akhurst and Boemare, 2000; Akhurst et al., 1996; Boemare et al., 1993; Suzuki et al., 1990) and 16S rDNA analyses (Brunel et al., 1997; Fischer-Le Saux et al., 1998; Liu et al., 1997; Suzuki et al., 1996; Szállás et al., 1997) validated the inclusion of these five species in, and the exclusion of *P. luminescens* from, the genus (see Taxonomy in this Chapter).

DNA/DNA hybridization analysis indicates that there are (to date) more than the five *Xenorhabdus* species. Data have been recorded for some of the strains that would be assigned to new *Xenorhabdus* species (Boemare and Akhurst, 1988; Bonifassi et al., 1999; Fischer-Le Saux et al., 1998), but too few to warrant a decision on their taxonomic status. A recent multivariate analysis of phenotypic data (Bonifassi et al., 1999) from *Xenorhabdus* strains (Fig. 10) confirmed that some strains should be assigned

to several new species (e.g., the symbionts of *S. arenarium*, *S. puertoricense*, *S. riobrave*, *S. scaptesci* and *S. serratum*) that will be defined in due course (Table 7).

**GENUS CHARACTERISTICS** Cells are Gram-negative, asporogenous, rod shaped, 0.3–2 µm by 2–10 µm and occasionally have filaments 15–50 µm in length. Spheroplasts, averaging 2.6 µm in diameter, appear in the last third of exponential growth. Proteinaceous crystalline inclusions develop in a large proportion of cells in stationary phase cultures. Cells move by means of peritrichous flagella, and swarming may occur on 0.6–1.2% agar. These bacteria are facultatively anaerobic, with both respiratory and fermentative types of metabolism. Optimum temperature is usually 28°C or less; a few strains grow at 40°C. Acid (no gas) is produced from glucose; fermentation of some other carbohydrates is poor. Strains are catalase negative, DNase positive and protease positive. Nitrate is not reduced to nitrite. Most tests used to differentiate Enterobacteriaceae are negative. Lipase is detected with Tween 20 and egg yolk agar; most strains are lipolytic on Tweens 40, 60, 80 and/or 85. Phase shift occurs to varying degrees in stationary phase cultures, giving rise to phase II cells that lack dye adsorption, antibiotic production, protein inclusions and some other characteristics of

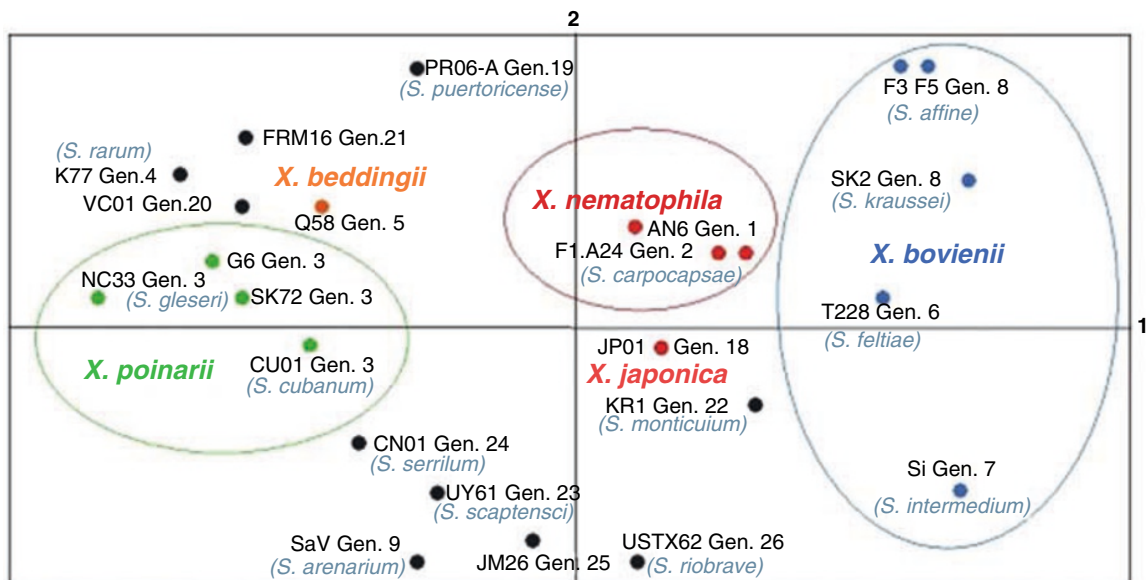


Fig. 10. Multivariate correspondence analysis of phenotypic data from *Xenorhabdus* strains. This Q-R reciprocal analysis used the software Statlab (SLP®) taking into consideration only the physiological and biochemical characters scored as variable. The strains are positioned in the A space defined by the two first components. Strain names with their PCR-RFLP genotype numbers (Fischer-Le Saux et al., 1998) are indicated at their position in the mathematical space. Those species represented by several strains are encircled. Host nematode species are indicated in blue between brackets for the isolated strains. Analysis of the inverted matrix (R analysis) shows that the first component is mainly supported by the growth-temperature variable, which is the reason “warm” strains of *Xenorhabdus* are on the left side while “temperate” strains are on the right side of the representation. Adapted from data published by Fischer-Le Saux et al. (1999a) and Bonifassi et al. (1999).

Table 7. Characteristics that differentiate *Xenorhabdus* species.

	<i>X. nematophila</i>		<i>X. bovienii</i>		<i>X. poinarii</i>		<i>X. beddingii</i>		<i>X. japonica</i>		<i>Xenorhabdus</i> sp.		<i>Xenorhabdus</i> sp.		<i>Xenorhabdus</i> sp.	
	<i>S. carpocapsae</i> ATCC19061 DSM3370	<i>S. feltiae</i> UQM2210 ATCC35271 DSM4766 6, 7, 8	<i>S. glaseri</i> UQM2216 ATCC35272 DSM4768	<i>S. longicaudum</i> (?) UQM2871 ATCC49542 DSM4764	<i>S. kushidai</i> IAM14265	<i>S. scapterisci</i> NR	<i>S. arenarium</i> NR	<i>S. sarratum</i> NR	<i>S. riobrave</i> NR							
16S rDNA genotype number	1, 2	6, 7, 8	3	5	18	23	9	24	26							
Number of strains	6	15	5	2	JP02	UY61	Sav	CN01	USTX62							
Number of strains or strain designation																
Simmons' citrate	+	+	+	+	-	+	+	-	-							
Esculin hydrolysis	-	-	d	+	-	+	+	-	-							
DNase	+	+	-	+	+	+	+w	-	+							
Phenylalanine deaminase	d	[-]	[-]	-	dw	NR	-	NR	NR							
Tryptophane deaminase	-	+	-	+w	-	-	-	-	-							
Indole	-	[-]	-	-	-	+	+	-	-							
Growth at 39–40°C	-	-	+	+	-	+	+	+	+							
Pigmentation	-	+	+	+	-	+	+	+	+							
Utilization of																
Citrate	+	[+]	+	+	-	-	+	-	-							
Diaminobutane	-	d	-	-	-	-	+	+w	-							
Fumarate	+	+	+	+	+	+	+	+	+							
D-Glucosamine	+	[+]	[+]	+	-	-	-	-	-							
DL-Glycerate	[+]	[+]	[-]	+	-	-	-	-	-							
L(-)Histidine	-	+	[+]	+	-	+	+	+	+							
myo-Inositol	+w	-	-	-	-	+	-	+w	+w							
DL-Lactate	+	+	+	+	+	+	+	+w	+w							
L(-)Malate	+	[+]	+	+	-	+	+	+w	+w							
Maltose	+	+	+	+	+	+	+	+	+							
Propionate	[-]	[-]	+w	+	-	-	-	-	-							
D(-)Ribose	[-]	[+]	-	+	-	+w	-	+w	+w							
Succinate	+	+w	+	+	-	+	+	+w	+w							
L-Tyrosine	-	[+]	-	+	-	-	-	-	-							

Symbols: +, 90–100% of strains are positive; [+], 76–89% are positive; d, 26–75% are positive; [-], 11–25% are positive; -, 0–10% are positive; w (e.g., [+w]) indicates a weak reaction.

Abbreviations of type strains: ATCC, American Type Culture Collection (Rockville, Md.); CIP, Collection de l'Institut Pasteur (Paris, France); <sup>T</sup>, type strain.

<sup>a</sup>Only five species have been defined in the genus. Several candidates are indicated in the columns 7–10 from which other companion strains are required.

<sup>b</sup>All tests were done at 28 ± 1 °C unless otherwise noted.

<sup>c</sup>Numbering of genotypes according to Fischer-Le Saux et al. (1998). (Modified from Fischer-Le Saux et al., 1999a.)

the phase I cells isolated from the natural environment. Biochemical identification of *Xenorhabdus* within the family Enterobacteriaceae is summarized in Table 7. The mol% G+C of the DNA is 43–50 (strain Bd). Strains are only found in the intestinal lumen of entomopathogenic nematodes of the family Steinernematidae and insects infected by these nematodes.

*XENORHABDUS NEMATOPHILA* The species characteristics of *Xenorhabdus nematophila* (ne.ma.tóphi.la. Modern entomological term *nematode*; Gr. adj. *phila*, loving or having affinity for; M.L. adj. *nematophila* nematode-loving; synonym: *Achromobacter nematophilus*; Poinar and Thomas, 1965; Thomas and Poinar, 1979) are listed in Table 7. No known isolates grow at temperatures in excess of 34°C. Neither phase I nor II colonies are pigmented. Most isolates are sensitive to furazolidone. Strains are found associated only with one species of nematode, *Steinernema carpocapsae*, but this association occurs around the world.

The mol% G+C of the DNA is 43–48 (strain Bd). Type strain number of the holotype is ATCC19061; paratype DSM3370. Genbank accession number of 16S rDNA is {D78009}.

*XENORHABDUS BOVIENII* The species characteristics of *Xenorhabdus bovienii* (bo.vi.en'i.i. M.L. gen.n. *bovienii*, of Bovien. Named for P. Bovien who first reported the presence of bacteria in the intestinal vesicle of a *Steinernema* species; Akhurst and Boemare, 1988) and *Xenorhabdus nematophilus* subsp. *bovienii* (Akhurst, 1983) are listed in Table 7. No growth occurs at 34°C; some strains will grow at 5°C. Strains are resistant to carbenicillin.

They are associated with several species of entomopathogenic nematode (*Steinernema feltiae*, *Steinernema intermedium*, *Steinernema kraussei* and *Steinernema affine*) in temperate regions.

The mol% G+C of the DNA is 44.3 (strain P1) and 46.9 (strain Bd). Type strain number of the holotype is UQM2210 (phase I of strain T228); paratype numbers are ATCC35271 and DSM4766. Genbank accession number of 16S rDNA is {X82254} and {D78007}.

*XENORHABDUS POINARII* The species characteristics of *Xenorhabdus poinarii* (poi.nar'i.i. M.L. gen.n. *poinarii*, of Poinar. Named for G.O. Poinar Jr. who made major contributions to the understanding of entomopathogenic nematode/bacterial interactions; Akhurst and Boemare, 1988) and *Xenorhabdus nematophilus* subsp. *poinarii* (Akhurst, 1983) are listed in Table 7. This is the most heat tolerant *Xenorhabdus*, with all strains growing at 36°C and some at 40°C. The intensity

of pigmentation in phase I varies from light to reddish brown. In some strains phase I cells do not produce antimicrobials; in some other strains, cells in both phases do.

In the United States, *X. poinarii* was first considered to be associated with only *Steinernema glaseri* (Akhurst, 1986b). A polyphasic approach, including phenotypic tests, restriction polymorphism analysis of PCR-amplified 16S rRNA genes, and DNA-DNA hybridizations with determination of the  $\delta T_m$ , was used to demonstrate that *S. cubanum* also harbors strains of *X. poinarii* (Fischer-Le Saux et al., 1999a). This bacterium is not pathogenic for most wax moth (*Galleria mellonella*) larvae unless associated with its nematode partner.

The mol% G+C is 42.6 (strain P1) and 49 (strain Bd). Type strain number of the holotype is UQM2216 (phase I of strain G); paratype numbers are ATCC35272, DSM4768. Genbank accession number of 16S rDNA is {X82253} (paratype DSM4768).

*XENORHABDUS BEDDINGII* The species characteristics of *Xenorhabdus beddingii* (bed.din'gi.i M.L. gen. N. *beddingii*, of Bedding. Named for R.A. Bedding who made significant contributions to the development of *Xenorhabdus/Steinernema* associations for insect pest control; Akhurst and Boemare, 1988), first named *Xenorhabdus nematophilus* subsp. *beddingii* (Akhurst, 1986a), are listed in Table 7. All isolates grow at 34°C, and some at 38°C. Cells hydrolyze esculin. They are inhibited by cephaloridine and ampicillin. The brown pigmentation is not strong. Phase I is highly unstable, producing the very stable phase II. They are associated with two undescribed species of *Steinernema* from Australia, one of which may be *Steinernema longicaudum*, a described species from China.

The mol% G+C of the DNA is 45.5–50 (strains Bd and P1). Type strain number of the holotype is UQM2871 (phase I of strain Q58); paratype numbers are ATCC49542, DSM4764. Genbank accession number of 16S rDNA is {X82254} (paratype DSM4764).

*XENORHABDUS JAPONICA* The species characteristics of *Xenorhabdus japonica* (ja.po'ni.ca. M.L. adj. *japonica*, of Japan; Nishimura et al., 1994) are listed in Table 7. Cells do not grow at 37°C. Arginine dihydrolase activity is detected in phase II. Pigmentation is yellowish brown. This species is only known to be associated with *Steinernema kushidai* in Japan.

The mol% G+C of the DNA is 45.9 (strain P1). Type strain number of the holotype is IAM14265. Genbank accession number of 16S rDNA is {D78008}.

## Habitat

*Xenorhabdus* species are insect pathogenic bacteria that occur naturally in the intestinal vesicle of nonfeeding infective stage entomopathogenic nematodes of the family Steinernematidae (Bird and Akhurst, 1983; Bovien, 1937; Poinar and Leutenegger, 1968; Fig. 11). After invading an insect host, the nematode commences development, releasing *Xenorhabdus* into the nutrient-rich hemolymph. The bacteria proliferate, killing the insect host and producing suitable nutrient conditions for nematode growth and reproduction, as well as an array of antibiotics and bacteriocins to minimize competition. As the nutrient source becomes depleted, the immature nematodes develop into dauer juveniles that will transport *Xenorhabdus* to a new nutrient source (Fig. 1; see Introduction in this Chapter).

### COSPECIATION BETWEEN THE BACTERIAL SYMBIONTS AND THEIR NEMATODE HOSTS

When the taxonomic data of *Xenorhabdus* is

compared with that of their host nematodes, a close relatedness of the two taxonomic structures is noticed, and a phenomenon of cospeciation between bacterium and nematode genera is shown (Table 8). However, sometimes several species of *Steinernema* can share one species of *Xenorhabdus*. For example, symbiotic species of *S. affine*, *S. feltiae*, *S. kraussei* and *S. intermedium* share *X. bovienii* (Akhurst and Boemare, 1988; Fig. 9), although specific 16S rDNA genotypes could be recognized for each one and subspecies may be defined soon. Two species of nematodes also were reported to harbor *X. beddingii*, but unfortunately the nematode species were not described at that time (Akhurst, 1986a). A recent report describes a similar observation for the symbionts of *S. cubanum* and *S. glaseri* (Fischer-Le Saux et al., 1999a). When *S. cubanum* was described, it was considered to be a related species of *S. glaseri* because some morphological characters were very similar (Mráček et al., 1994). These morphological similarities make it difficult to distinguish the two species (Hominick

Fig. 11. *Steinernema*, nematode host of *Xenorhabdus*, and anatomical localization of the phoretic bacteria. (a) Scanning electron micrograph of dauer juveniles. Bar = 100  $\mu\text{m}$ . (b) Scanning electron micrograph of dauer juveniles. Bar = 10  $\mu\text{m}$ . (c) Scanning electron micrograph of dauer juveniles. Detail is shown of the ornamentation of the cuticle. Bar = 2  $\mu\text{m}$ . (d) Scanning electron micrograph of dauer juveniles. Bar = 1  $\mu\text{m}$ . Detail showing the closed mouth. (e) Scanning electron micrograph of dauer juveniles. Exsheathing of the old cuticle is in progress. To eliminate all the contaminants between the old and new cuticles (Fig. 11f), dauer juveniles must be removed from their old L2 cuticle during axenization. Bar = 5  $\mu\text{m}$  (from Bonifassi et al., 1999). (f) Microphotograph of native *Steinernema scapterisci* tail. Light microscopic image, using Nomarski differential interference contrast optics, shows microorganisms (arrows) between L2 and L3 cuticles before the exsheathing (arrows). Bar = 20  $\mu\text{m}$  (from Bonifassi et al., 1999). (g) Photomicrograph of the anterior part of the intestine of a dauer larva of *Steinernema feltiae* showing the vesicle containing *Xenorhabdus bovienii* symbionts. Bar = 5  $\mu\text{m}$  (from Bird and Akhurst, 1983).

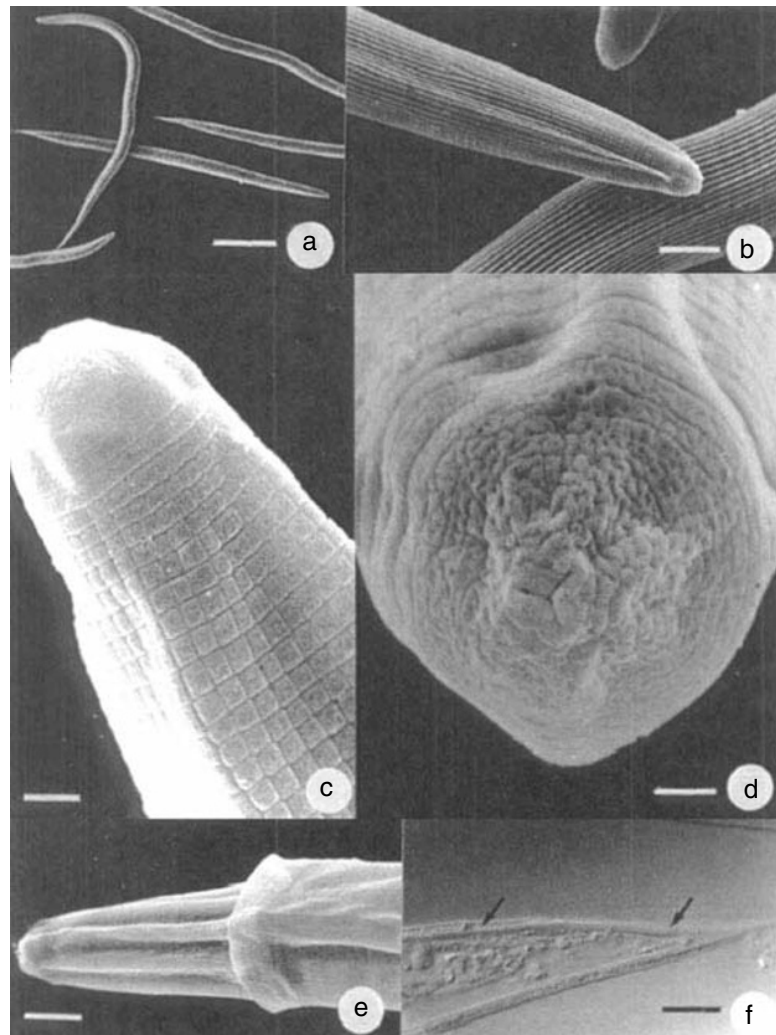


Table 8. Species correspondence between *Xenorhabdus* and *Steinernema* nematodes.

<i>Steinernema</i>	Symbiont	Secondary guest
<i>S. krausset</i> (type species)	<i>Xenorhabdus bovienii</i>	
<i>S. abbas</i>	<i>Xenorhabdus</i> sp.	<i>Pseudomonas</i> (= <i>Flavimonas</i> ) <i>oryzthabitans</i> <sup>1</sup>
<i>S. arenarium</i> (synonym: <i>S. anomatae</i> )	<i>Xenorhabdus</i> sp.	
<i>S. affine</i>	<i>Xenorhabdus bovienii</i>	
<i>S. bicomutum</i>	<i>Xenorhabdus</i> sp.	
<i>S. carpocapsae</i>	<i>Xenorhabdus nematophila</i>	<i>Alcaligenes, Pseudomonas, Acinetobacter</i> <sup>2</sup>
<i>S. cubamon</i>	<i>Xenorhabdus poinarii</i>	
<i>S. feltiae</i>	<i>Xenorhabdus bovienii</i>	
<i>S. glaseri</i>	<i>Xenorhabdus poinarii</i>	
<i>S. intermedium</i>	<i>Xenorhabdus bovienii</i>	
<i>S. kushidai</i>	<i>Xenorhabdus japonica</i>	
<i>S. longicaudum</i> (?)	<i>Xenorhabdus beddingii</i> <sup>3</sup>	
<i>S. monticulum</i>	<i>Xenorhabdus</i> sp.	
<i>S. puertoricense</i>	<i>Xenorhabdus</i> sp.	
<i>S. rarum</i>	<i>Xenorhabdus</i> sp.	
<i>S. riobrave</i>	<i>Xenorhabdus</i> sp.	
<i>S. scapterisci</i>	<i>Xenorhabdus</i> sp.	<i>Ochrobactrum, Paracoccus, Xanthomonas</i> <sup>4</sup>
<i>S. serratum</i>	<i>Xenorhabdus</i> sp.	

et al., 1997). The restriction analysis of the intergenic transcribed spacer (ITS) of the ribosomal genes revealed high molecular similarities between *S. cubanum* and *S. glaseri* (Hominick et al., 1997), and the construction of a dendrogram illustrated their close phylogenetic relatedness (Reid and Hominick, 1998). A recent common ancestor could explain high morphological and molecular similarities between these two nematode species. The geographical isolation of the fauna from Cuba could have led to their speciation, which may be relatively recent. Although the bacterial symbionts of the two nematodes at present belong to the same species, the phenomenon of cospeciation may be in progress, and phenotypic and genotypic bacteriological discriminations are not sufficient to delineate two *Xenorhabdus* species. On the basis of these results, one can assume that the previously mentioned exceptions do not fundamentally modify the concept of a possible coevolution.

Questions similar to those asked about *Photorhabdus* (see Cospeciation in this Chapter) are: how do the bacteria and the nematodes maintain their specific association? signal compounds for recognition? specific attachment? In the case of *Xenorhabdus*, preservation of the bacterial cells from the digestion of the dauer larvae during the free-living phase of the host's life cycle is a function of the nematode's anatomy, i.e., special vesicle in the intestine of the dauer (Bird and Akhurst, 1983; Fig. 11g). This seems to be a clear coevolutionary trait, not appearing in other free-living soil-dwelling nematodes such as species of the family Rhabditida. It may be reasonable to hypothesize that specific attachment by the symbiotic bacteria to such an organ is the mechanism ensuring the maintenance of the symbiosis.

## Isolation

*Xenorhabdus* spp. generally grow well at 25–28°C on nutrient or similar agar (e.g., Luria-Bertani, trypticase soy). Like *Photorhabdus* (see Isolation in this Chapter), *Xenorhabdus* can be isolated from the infective stage nematodes by the hanging drop technique or by maceration (Akhurst, 1980; Poinar, 1966a). For both methods the infective juveniles must first be surface sterilized; this is readily achieved by immersing a small number of live infective stage nematodes (>100), free of debris, in 0.1% merthiolate for 1 h at room temperature and then rinsing thoroughly in several changes of sterile water. In the hanging-drop technique, individual surface-sterilized infective juveniles are transferred to a drop of sterile change by aseptically collected insect hemolymph on a cover slip that is then inverted over a cavity to prevent desiccation. The drop is incubated (1–3 d) at 25°C until the nematodes commence development. At this time, they void their symbiotic bacteria, which can be subcultured from the hemolymph onto an agar medium (e.g., nutrient agar) 1 d later. A more rapid method involves the maceration by means of a tissue homogenizer of 50–100 surface-sterilized infective juveniles in a nutrient broth. The macerated nematodes (in 10–100 µl aliquots) should be plated onto an agar medium immediately and incubated at 28°C for 3 d. The inclusion of suitable controls to confirm that the surface-sterilization procedure has been effective is essential for both methods. *Xenorhabdus* can also be isolated by the less labor-intensive method of collecting hemolymph from an insect (e.g., *G. mellonella*) larva within 24 h of its death caused by *Steinernema*. With this last method, bacteria



other than *Xenorhabdus* may also be isolated; these bacteria may be carried into the host on the exterior of the nematodes or may be picked up into the hemolymph from the insect cuticle. Contamination by other bacteria can be minimized by burying the insect in clean, damp sand, adding a small number of nematodes to the surface of the sand and incubating at 20–25°C until 1–5 nematodes infect the insect. This last method is better suited to re-isolation of a *Xenorhabdus* strain rather than to identification of the bacteria specifically associated with a nematode species.

Identification

As discussed for *Photorhabdus* (see Isolation), the use of 16S rDNA probes may be useful to identify the *Xenorhabdus* species (Fig. 9). Interestingly, several PCR-RFLP 16S rRNA genotypes (Fig. 9; Table 7) can be recognized inside a DNA-DNA relatedness group that may be useful to distinguish subspecies that correlate with the nematode host species (*N. Boemare*, unpublished observation). This is the case of genotypes 6,7 and 8 inside the *X. bovienii* genomic group (Brunel et al., 1997), which can be more or less related to symbionts of *S. feltiae*, *S. kraussei* and *S. affine* that are not yet distinguishable by DNA-DNA hybridizations (Figs. 9 and 10). If such an approach can be accurately verified, it will provide an amazing result since DNA-DNA hybridization is considered the gold standard method for defining species and subspecies, whereas the ribosomal methods are useful for the genera.

*Xenorhabdus* are easily distinguished from other Enterobacteriaceae by the absence of catalase and their inability to reduce nitrate. The key characteristics for differentiating *Xenorhabdus* from *Photorhabdus* are luminescence and

catalase. The major cellular fatty acids of *Xenorhabdus* are C<sub>16:0</sub>, C<sub>16:1</sub>, C<sub>18:1</sub> and C<sub>17-cy</sub>, and the respiratory quinone system is ubiquinone-8 (Suzuki et al., 1990). Antibiograms scored after 3 d at 28°C show that *Xenorhabdus* are inhibited by streptomycin, neomycin, gentamycin, tetracycline, kanamycin and colistin, but not penicillin. Most strains are resistant to ampicillin and cephaloridine, and to a lesser extent, furazolidone, whereas resistance to chloramphenicol is limited. Resistance to streptomycin, tetracycline and kanamycin after selection has been demonstrated for *X. nematophila*.

Insect pathogenicity is tested by injection of 10<sup>2</sup> and 10<sup>4</sup> cells (total count) from a 24-h broth culture into final instar *G. mellonella* larvae. The injected larvae should be placed on dry filter paper in Petri dishes and incubated at 25°C for 3 d. Most *Xenorhabdus* spp. will kill <50% at a dosage of 10<sup>2</sup> cells; all will kill <50% at 10<sup>4</sup> cells.

Cultivation

*Xenorhabdus* species are easily grown in vitro on a range of complex liquid and solid media and in minimal media supplemented with nicotinic acid, *p*-aminobenzoic acid, serine, tyrosine, and/or proline (Grimont et al., 1984). Minimal medium II (BioMèrieux®) contains all the necessary requirements to test utilization of organic compounds. Nutrient agar is suitable for all strains. They are mesophilic; most grow between 15 and 30°C, but strains growing at 4 or at 40°C have been isolated. All tests for phenotypic characterization of *Xenorhabdus* should be conducted at 28°C. Dye adsorption in most *Xenorhabdus* species can be tested on nutrient agar containing 0.0025% (w/v) bromthymol blue and 0.004% (w/v) triphenyltetrazolium chloride

Table 9. *Xenorhabdus* 16S rDNA sequences.<sup>a</sup>

Accession	Strain	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	Ref.
X82251	DSM4768	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	G	A	G	G	G	C	T	T	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	5	
D78009	ATCC19061	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	G	A	G	G	G	C	T	T	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	6	
Z76738	<i>Xenorhabdus nematophila</i>	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	G	A	G	G	C	T	T	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	7; 4		
Z76737	mex.str.N2-4	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	G	A	G	G	C	T	T	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	7		
Z77209	103 Nealson	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	G	A	G	G	C	T	T	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	7		
D78008	IAM14265	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	G	A	G	G	C	T	T	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	6		
Z76739	SK-1J	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	G	A	G	G	C	T	T	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	7		
D78006	DSM4764	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	G	A	G	G	C	T	T	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	6; 4		
X82254	DSM4764	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	G	A	G	G	C	T	T	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	5		
D78010	DSM4768	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	N	A	N	C	C	T	G	A	A	T	A	G	G	T	T	G	G	C	G	T	T	G	A	C	G	T	T	A	C	C	C	6; 4			
X82253	DSM4768	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	N	A	N	C	C	T	G	A	A	T	A	G	G	T	T	G	G	C	G	T	T	G	A	C	G	T	T	A	C	C	C	5			
D78007	ATCC35271	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	T	A	G	G	C	T	T	G	T	G	A	T	T	G	A	C	G	T	T	A	C	C	C	6; 4		
X82252	DSM4766	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	T	A	G	G	C	T	T	G	T	G	A	T	T	G	A	C	G	T	T	A	C	C	C	5		
Z77212	Sulc.strain	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	T	A	G	G	C	T	T	G	A	T	T	G	A	C	G	T	T	A	C	C	C	7				
U70319	S.felB1	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	T	A	G	G	C	T	T	G	A	T	T	G	A	C	G	T	T	A	C	C	C	3				
Z77210	S.F.22	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	T	A	G	G	C	T	T	G	A	T	T	G	A	C	G	T	T	A	C	C	C	7				
U70318	S.intB	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	T	A	G	G	C	T	T	G	A	T	T	G	A	C	G	T	T	A	C	C	C	3				
Z77211	Norv.	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	T	A	G	G	N	T	G	T	G	A	T	T	G	A	C	G	T	T	A	C	C	C	7			
AF079816	XenoOS21	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	T	A	G	G	T	T	G	T	G	A	T	T	G	A	C	G	T	T	A	C	C	C	3			
Z77213	Filip	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	T	A	G	G	T	T	G	T	G	A	T	T	G	A	C	G	T	T	A	C	C	C	7			
U70320	S.rioB	C	A	G	C	G	G	G	A	G	G	A	A	G	G	C	G	T	A	A	G	T	C	T	G	A	A	C	A	C	G	G	C	T	C	A	C	G	A	T	T	G	A	C	G	T	T	A	C	C	C	3	

<sup>a</sup>The variable region with its conserved flanking parts is only represented from positions 440 to 490 (*E. coli* numbering). Data collected from gene databases (updated April 2000) have been selected and aligned with the aid of algorithms BLAST (Altschul et al., 1997) and CLUSTAL (Thompson et al., 1997). References: (1) Liu et al., 1997; (2) Pütz et al., 1990; (3) Rainey et al., 1995; (4) Suzuki et al., 1996; (5) Szállás et al., 1997.

(Figs. 12a, b). Dye adsorbing phase I colonies of *X. nematophila* will appear dark blue, of *X. bovienii* and other yellow pigmented strains tend to be green, and of *X. beddingii* are more maroon than blue because they do not adsorb so strongly; nonabsorbent phase II colonies in all of these are red (Akhurst, 1980). As *X. poinarii* does not adsorb bromthymol blue, dye adsorption in this species can be assessed on MacConkey agar; adsorbing colonies are dark red (Akhurst, 1986b; Boemare and Akhurst, 1988). Congo red (Francis et al., 1993), and some other dyes can also be used for most *Xenorhabdus* spp. Antibiotic production of phase I variants is tested by spot-inoculating *Xenorhabdus* onto nutrient agar and incubating at 28°C for 3 d. After this time the bacteria are killed by exposure to chloroform vapor for ca. 1 h. After the chloroform has evaporated from the agar, semi-solid nutrient agar (0.5%) inoculated with a suitable indicator organism (*Micrococcus luteus* or another Gram-positive species) is poured to form a thin layer. After incubation at 28°C overnight, a halo of inhibition around antibiotic-producing colonies will be evident.

### Preservation

Storage in 20% glycerol at temperatures at -70°C or below is very useful for long-term maintenance of *Xenorhabdus* cultures; they do not store well at -20°C. Short-term storage (less than one month) is best conducted at 10–15°C because survival on agar or in broth at 4°C is very poor and when cultures are maintained at temperatures in excess of 15°C, the risk of a proportion of the culture undergoing phase change is significant.

A remarkable feature must be noted. Nematodes of the species *S. feltiae*, kept in collection for more than 10 years, preserve their symbionts; bacterial strains isolated after a such delay were identical, in terms of phenotypic and genotypic characters, to those initially isolated (N. Boemare and F. Grimont, personal communication). Consequently, when the conditions of storage of the nematodes are good, reisolation of the *Xenorhabdus* strain from their dauer larvae may be advised for those species where the association is strongly observed. This is not the case for the *S. glaseri* and *X. poinarii* association, which seems to be more labile.

### Physiology

**PHASE VARIATION** Phase I *Xenorhabdus*, such as those isolated from the nematode's associates or insects infected by the nematodes, form convex, circular colonies with slightly irregular margins and a diameter of 1.5–2 mm after 4 d at 28°C;

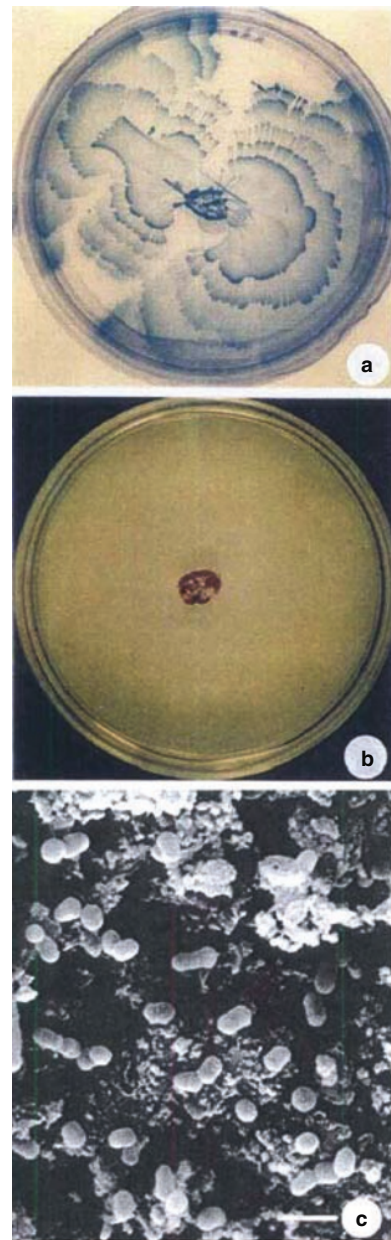


Fig. 12. Motility and adhesion of *Xenorhabdus nematophila*. (a) Behavior of *X. nematophila* F1/1 variants on 1% NBTA agar (24-h-old colony). F1/1 cells induce a swarming colony when inoculated at the center of plates containing 0.6 to 1.2% agar. On 1% agar, periodic cycles of migration (clear zones) and cell division, also named “consolidation,” (dark zones) are easily visualized (Givaudan et al., 1995). In addition adsorption of bromthymol blue on NBTA (blue colony), is a characteristic of phase I variants. (b) In contrast phase II variants show a complete lack of swarming motility (Givaudan et al., 1995), do not adsorb the bromthymol blue, but reduce the 2,3,5-triphenyltetrazolium chloride (TTC) to formazan (red colony). (c) Scanning electron micrograph of the colonization by *Xenorhabdus nematophila* strain F1 on sponge substrate coated with the nutrient medium of Wouts (1982), shows adhesion of the cells on solid substrate. Bar = 1 µm.

they also have a slightly granular appearance and, in some species, are pigmented (yellow, brown). These colonies adsorb dyes, such as bromthymol blue and neutral red, taking on intense coloration (Fig. 12a, b). Phase II variants generally form similar colonies but flatter and wider (diameter 2.5–3.5 mm after 4 d at 28°C) and with lesser pigmentation; these colonies adsorb dyes only very weakly and no coloration is evident in 4-d colonies. Phase I cells have peritrichous flagella responsible for the motility of the strain (Givaudan et al., 1995) expressed essentially in nutrient broth (Fig. 13a). Phase I, but not phase II, cells have a peritrichous array of fimbriae expressed on solid nutrient agar (Fig. 13b) with diameter of 6.4 nm, morphologically similar to the type I fimbriae of *E. coli* (Binnington and

Brooks, 1994; Brehélin et al., 1993; Moureaux et al., 1995). The glycocalyx surrounding *Xenorhabdus* cells is irregular in thickness, with a mean depth of 142 nm and 49 nm in phase I and phase II *X. nematophila*, respectively (Brehélin et al., 1993; Fig. 13c). Both fimbriae and glycocalyx are presumably responsible for the cell adhesion on substrate (Fig. 12c). Phase I cells produce during the stationary period crystalline protoplasmic inclusions characteristic of the genus (Fig. 14b, d).

With some strains of *X. nematophila*, and depending on the insect tested, phase II variants may be less pathogenic than the phase I variants. Thus an analysis of pathogenicity in *Manduca sexta* larvae revealed that the phase II variant of AN6 was significantly less virulent than the phase I variant (Völgyi et al., 1998). The phase II variant of *X. nematophila* strain N2-4 was also avirulent for *M. sexta*. On the other hand, F1/II and 19061/II were as virulent as their respective phase I cells. Consequently phase traits of *Xenorhabdus*, which are mainly involved in the growth and development of nematodes, may be considered as “symbiotic characters,” and to some extent depending on the biotic conditions of the infection (infecting strain and susceptibility of the insect), more or less linked with virulence factors. Nevertheless the role of phase II remains uncertain in *Xenorhabdus*, as in *Photorhabdus*.

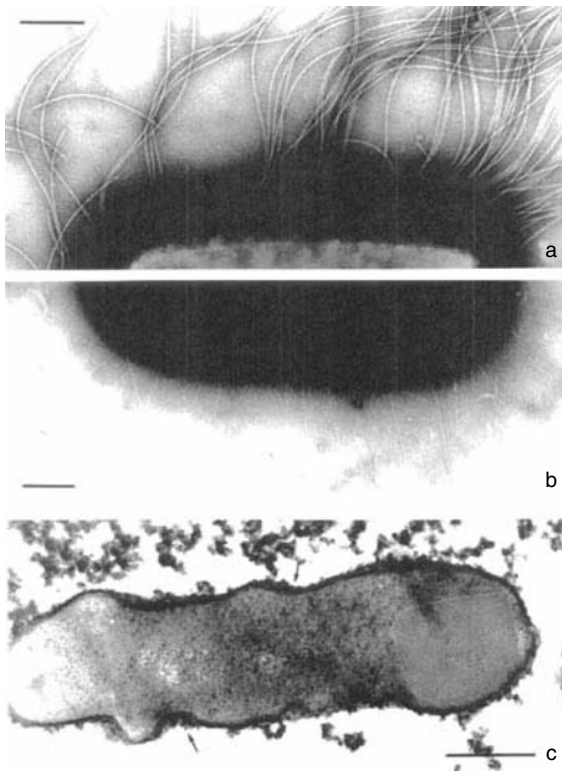


Fig. 13. Surface appendages of *Xenorhabdus nematophila* phase I variants. (a) Transmission electron micrograph of F1/1 cell from nutrient broth culture, showing peritrichous flagella; negative staining with 1% phosphotungstate. Bar = 0.5  $\mu\text{m}$ . (b) Transmission electron micrograph of F1/1 cells from nutrient agar at 1.5% previously dried to minimize expression of flagella, showing fimbriae (or pili) around the cell wall. Bacteria were fixed 1.25% glutaraldehyde and 1% paraformaldehyde in 0.015M sodium cacodylate buffer (pH 7.4; 10 min) and were negatively stained with 0.5% phosphotungstate (from Moureaux et al., 1995). Bar = 0.25  $\mu\text{m}$ . (c) Transmission electron micrograph of A24/1 cell grown on nutrient agar. Cell fixed with glutaraldehyde-lysine-osmium tetroxide and stained with ruthenium red, uranyl acetate and lead citrate shows significant glycocalyx surrounding the cell wall (arrows). Bar = 0.5  $\mu\text{m}$  (from Brehélin et al., 1993).

SWARMER CELLS Givaudan et al. (1995) demonstrated that *Xenorhabdus* phase I variants displayed a swarming motility when they were grown on a suitable solid medium (0.6–1.2% agar; Fig. 12a, b). Unlike most phase I variants of different *Xenorhabdus* spp., phase II variants were unable to undergo cycles of rapid and coordinated population migration (swarm and even to swim) over the surface of semisolid agar, particularly those of *X. nematophila*. Optical and electron microscopic observations showed that the nonmotile phase II cells of *X. nematophila* F1 lacked flagella. When flagella from strain F1 phase I variants (Fig. 13a) were purified, the molecular mass of the flagellar structural subunit was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 36.5 kDa. Flagellin from cellular extracts or culture medium of phase II cells was undetectable with antiserum against the denatured flagellin by immunoblotting analysis. This suggests that the lack of flagella in phase II cells is due to a defect during flagellin synthesis as has been demonstrated by genetic studies. (See Genetics in this Chapter)

PRODUCTION OF SECONDARY METABOLITES As for *Photorhabdus*, phase I variants of *Xenorhabdus*

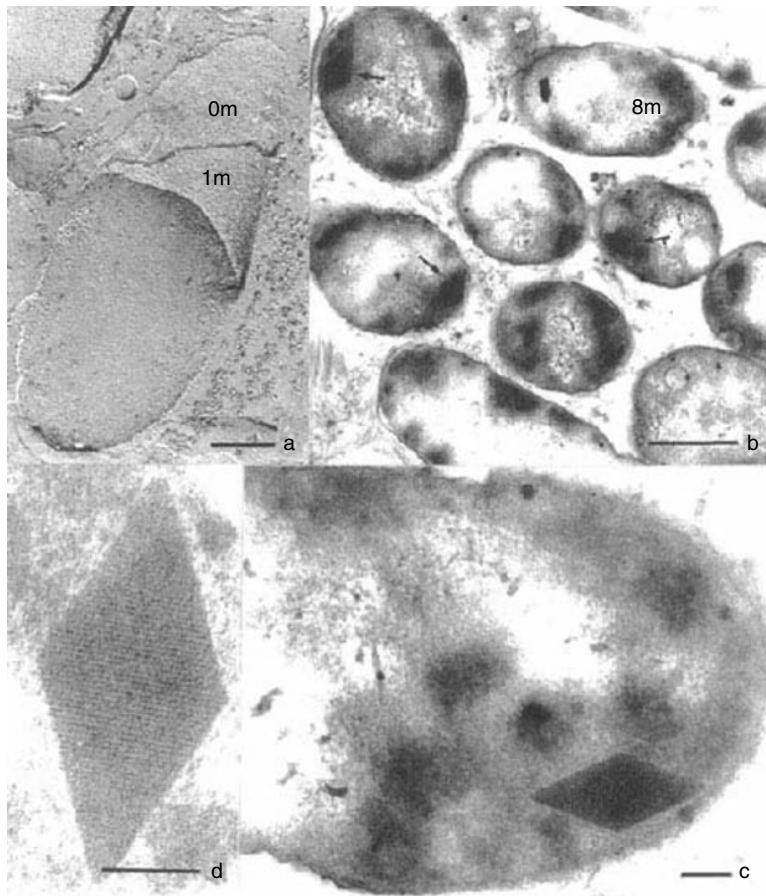


Fig. 14. Ultrastructural traits of *Xenorhabdus nematophila* cells. (a) Freeze-etching of strain F1 cell showing the outer (om) and the inner (im) membranes of the cell wall. Cleavage faces have been displayed, both at the level of cell wall and of plasma membrane. Glutaraldehyde (2.5%) in 0.1 M cacodylate buffer (pH 7.2) was added at 37°C for 1 h. The flask was rinsed with the cacodylate buffer at laboratory temperature, and the cells were gently scraped. The cells were sedimented by low-speed centrifugation, and the pellet was impregnated with 25% glycerol for 20 to 30 min to avoid ice-crystal formation. These samples were again centrifuged, and then were sandwiched between two specimen-holder dishes 3 mm in diameter, in preparation for the double-replica technique. They have been fractured in a Cryofract and both complementary fractures were shadowed by platinum evaporation. The replicas were cleaned in sodium hypochloride, rinsed in double distilled water, dried, and examined in a transmission electron microscope. Bar = 0.4 µm. (b) Cultures of 5-d stationary growth of strain F1 fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, and stained with uranyl acetate and lead citrate. Crystalline inclusions (arrows) begin by a condensation of amorphous material (am), which crystallizes as rhomboedric bodies. Bar = 0.8 µm. (c) Same as b, except bar = 0.1 µm. (d) Same as c but shows details of the crystallization of the inclusion. Bar = 0.1 µm.

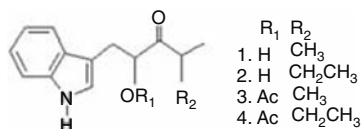
produce a variety of secondary metabolites, some of which have antimicrobial properties (Akhurst, 1982a; Table 10). Four chemical groups have been characterized: indole derivatives (Li et al., 1996; Li et al., 1997; Paul et al., 1981; Sundar and Chang, 1993; Webster et al., 1996), xenorhabdins (McInerney et al., 1991a; Rhodes et al., 1987), xenorixides (oxidized xenorhabdins) and xenocoumacins (Gregson and McInerney, 1989; McInerney et al., 1991b). Maxwell et al. (1994) examined occurrence of the xenocoumacins in vivo (in the insect *Galleria mellonella*) and in vitro (in tryptic soy broth). The level of antibiotic activity was greater under in vivo than in vitro conditions. These experi-

ments demonstrate that *Xenorhabdus* antibiotics may play an important role by preventing microbial contamination in the insect carcass during the development of nematodes. The production of antimicrobials provides at least a partial explanation for the “natural monoxeny” during parasitism.

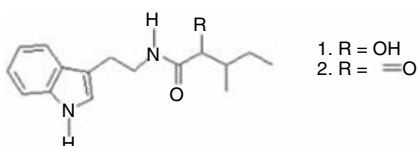
**LYSOGENY AND BACTERIOCINOGENY** The presence of lysogenic phage in and production of bacteriocins by *X. nematophila* was demonstrated by Boemare et al. (Boemare et al., 1992; Fig. 15). Lysis of either phase of *X. nematophila*, *X. bovienii* or *X. beddingii* in response to mitomycin C or heat shock released complete and partial

Table 10. Secondary antibiotic metabolites from *Xenorhabdus* strains.

## 1. Indole derivatives



R1, R2, substituents	<i>in vitro</i> production	active against
3-(2'-hydroxy-3'-keto-4'-methylpentyl)-indole	+	bacteria
3-(2'-hydroxy-3'-keto-4'-methylhexyl)-indole	+	bacteria
3-(2'-acetoxy-3'-keto-4'-methylpentyl)-indole	+	bacteria
3-(2'-acetoxy-3'-keto-4'-methylhexyl)-indole	+	bacteria



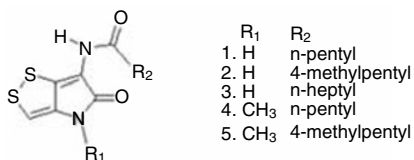
R substituents	<i>in vitro</i> production	active against
3-indoleethyl(3'-methyl-2'-hydroxy)pentamide	+	—
3-indoleethyl(3'-methyl-2'-oxo)pentamide (nematophin)	+	bacteria, fungi

Species: *X. nematophila* ATCC 19601, B1, AII

*X. bovienii* DN, B1, BC1, D1

(Li et al., 1996; Li et al., 1997; Paul et al., 1981; Sundar and Chang, 1993; Webster et al., 1996)

## 2. Xenorhabdins

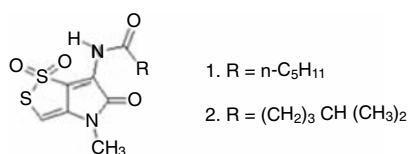


N-acyl derivatives of	<i>in vitro</i> production	Active against
6-amino-4,6-dihydro-6-oxo-1,2-dithiolo(4,3-b)pyrrole	+	bacteria, insects
6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo(4,3-b)pyrrole	+	bacteria, insects

Species: *Xenorhabdus bovienii*

(McInerney et al., 1991; Rhodes et al., 1987)

## 3. Xenorxides

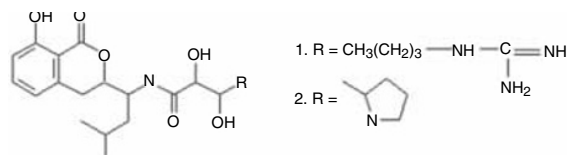


Derivatives of	<i>in vitro</i> production	active against
oxidized xenorhabdins	+	bacteria, fungi

Species: *Xenorhabdus bovienii*

(Li et al., 1997)

## 4. Xenocoumacins



Derivatives of	<i>in vitro</i> production	active against
3,4-dihydro-8-hydroxy-1H-2-benzopyran-1-one	+	Stress-induced ulcers, bacteria, fungi

Species: *Xenorhabdus nematophila*

(Gregson and McInerney, 1989; McInerney et al., 1991)

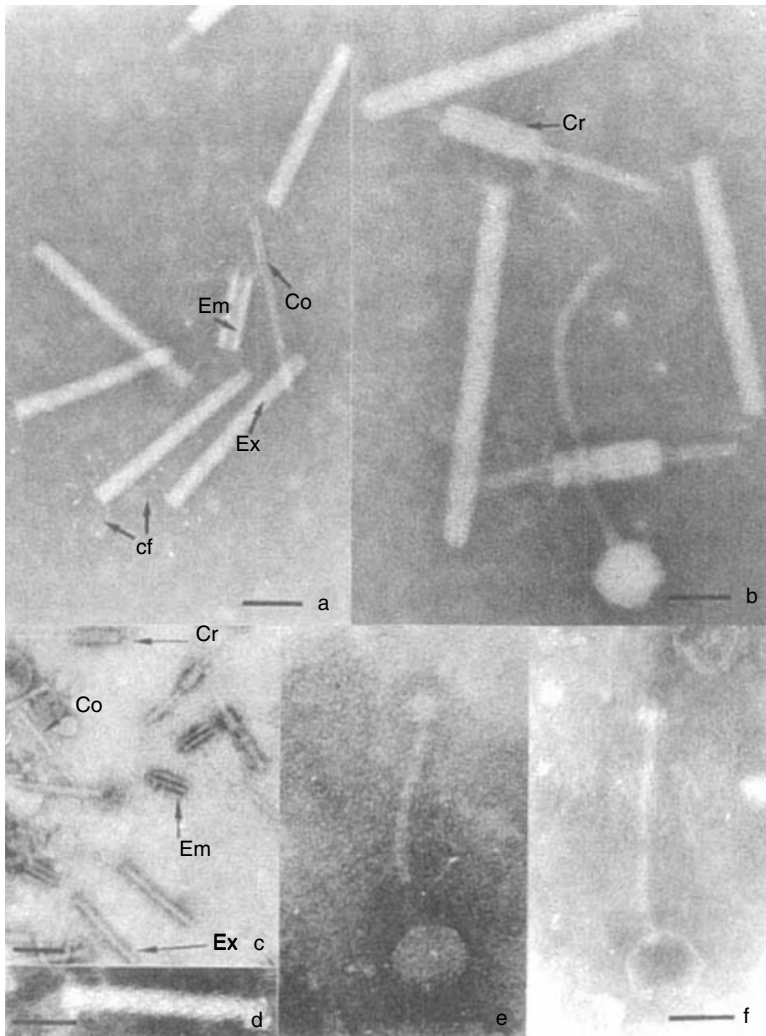


Fig. 15. Electron microscopy of phages (belonging to the family Siphoviridae) and bacteriocins (xenorhabdycin) of *Xenorhabdus nematophila*. Mitomycin C or heat shock treatments induce a phage production at the origin of the culture lysis. At the same time, the production of bacteriocin particles is highly increased. (a) Purified xenorhabdycin particles from mitomycin C-induced culture of strain F1. Bacteriocins with extended sheath (Ex) and caudal fibers on baseplate with adhesive extremities (cf), empty bacteriocins (Em), and loose cores (Co). Bar = 50 nm. (b) Phage and bacteriocin particles in the F1 strain lysate. Cr = bacteriocin with contracted sheath. Bar = 30 nm. (c) Mixture of the different particles of a purified xenorhabdycin suspension of strain A24. Same abbreviations as those in legend to panels 14a and 14b. Bar = 100 nm. (d) Detail showing the surface striation of a complete xenorhabdycin particle of strain A24. Same abbreviations as those in legend to panels 14a and 14b. Bar = 50 nm. (e) Detail of a complete phage of strain A24. Bar = 50 nm. (f) Detail of an A24 phage with empty head. Bar = 50 nm (from Boemare et al., 1992; Baghdiguian et al., 1993; Thaler et al., 1995).

phages. The phage of *X. nematophila* belongs to the family Siphoviridae, as does the  $\lambda$  phage from *E. coli*. The phage head capsid has one major and two minor subunits (Thaler et al., 1995).

The bacteriocins are phage tail-like particles, which in addition to inhibiting non-host *Xenorhabdus*, inhibited *P. luminescens*, *Proteus vulgaris* and *Morganella morgani*. However, none of the other Gram-negative or Gram-positive bacteria tested were inhibited, indicating that the bacteriocins act against closely related genera, in contrast to the antibiotics produced by phase I variants that have a wide spectrum of activity (Boemare et al., 1992; Table 11). Electron microscopic studies of the *X. nematophila* bacteriocin, named xenorhabdycin (Thaler et al., 1995), show that bacteriocin particles have the structure of a rigid phage tail without any "head" (Baghdiguian et al., 1993; Fig. 15). Biochemically, xenorhabdycin was shown to consist of two major protein bands, corresponding to the sheath and core, and five minor bands (Thaler et al., 1995).

The inhibition by several dilutions of a xenorhabdycin suspension was scored 24 h after deposit on a lawn of the bacterial target (+, clear zone at the droplet site, -, no clear zone). The bacteriocin suspension was purified from a culture of *X. nematophila* A24 ( $A_{600} = 0.5$ ) after induction by mitomycin C or heat shock and was diluted up to  $10^{-5}$  (Thaler et al., 1995) before assessing bacteriocin activity. When noninduced cultures were centrifuged and the filter-sterilized supernatants were tested similarly, a residual bacteriocin activity was noted for the same susceptible target strains of this table, meaning that a spontaneous bacteriocinogenesis occurs in untreated cultures, albeit at a lower frequency than in induced cultures (Boemare et al., 1992). The xenorhabdycin producer strain and strains belonging to the same species are immune from this antibiotic activity (blue box in the table), strains of other species of *Xenorhabdus* and *Photorhabdus* are susceptible (red box), and the only other genera containing susceptible strains are *Proteus* and *Morganella* (pink box), showing that

Table 11. Antibiotic activity of xenorhabdycin from both variants of *X. nematophila* strain A24.

Indicator strains		Growth inhibition
<i>Citrobacter freundii</i>		–
<i>Enterobacter cloacae</i>		–
<i>Erwinia chrysanthemi</i>		–
<i>Klebsiella pneumoniae</i>		–
<i>Salmonella typhimurium</i>		–
<i>Serratia marcescens</i>		–
<i>Yersinia enterocolitica</i>		–
<i>Pseudomonas aeruginosa</i>		–
<i>Pseudomonas testosteroni</i>		–
<i>Micrococcus luteus</i>		–
<i>Micrococcus roseus</i>		–
<i>Streptococcus epidermidis</i>		–
<i>Streptococcus faecalis</i>		–
<i>Bacillus cereus</i>		–
<i>Bacillus megaterium</i>		–
<i>Bacillus subtilis</i>		–
<i>Bacillus thuringiensis</i>		–
<i>Escherichia coli</i>		–
<i>Proteus mirabilis</i>		–
<i>Morganella morganii</i>		+
<i>Proteus vulgaris</i>		+
<i>Photorhabdus luminescens</i>	K80/1	+
<i>Photorhabdus luminescens</i>	K80/2	+
<i>Xenorhabdus beddingii</i>	Q58/1	+
<i>Xenorhabdus beddingii</i>	Q58/2	+
<i>Xenorhabdus nematophila</i>	F1/1	–
<i>Xenorhabdus nematophila</i>	F1/2	–
<i>Xenorhabdus nematophila</i>	A24/1	–
<i>Xenorhabdus nematophila</i>	A24/2	–

this bacteriocin is active only against species closely related to *X. nematophila*.

## Genetics

Genetic studies (comprehensively reviewed by Forst and Neilson, 1997; Forst et al., 1996) have been undertaken to analyze the mechanisms and functions of pathogenicity, symbiosis and phase variation. Couche et al. (1987b) demonstrated the presence of one or two small plasmids (3.6–12 kb) in some, but not all, *X. nematophila* and *X. bovienii* strains. The plasmid profiles did not differ between phases I and II. Smigielski and Akhurst (1994a) reported two megaplasmids (71.8 and 118.5 kb) as well as two additional plasmids (6.5 and 17 kb) in the A24 strain of *X. nematophila*. They also demonstrated that all strains of *X. nematophila*, *X. bovienii*, *X. beddingii* and *X. poinarii* contained megaplasmids (48–680 kb) and that the megaplasmid profiles of phases I and II were not different. Small or large plasmids of *Xenorhabdus* have not been further characterized or engineered to generate cloning vectors.

Conjugation of plasmids from *E. coli* has been applied successfully for *X. nematophila* and *X. bovienii* (Forst and Neilson, 1996; Francis et al.,

Table 12. Identified genes and proteins from *Xenorhabdus* strains.

Gene	Protein encoded	% identity	with
<i>opnP</i> (1)	porin	59	<i>E. coli</i>
<i>ompR</i> (4)	cytoplasmic regulatory protein	78	<i>E. coli</i>
<i>envZ</i> (4)	osmolarity sensor membrane protein	57	<i>E. coli</i>
<i>fliC</i> (2)	flagellin	68	<i>E. coli</i>
<i>fliD</i> (2)	hook-associated protein	41	<i>E. coli</i>
<i>fliH</i> (3)	master regulator	78	<i>E. coli</i>
<i>fliI</i> (3)	master regulator	72	<i>E. coli</i>
<i>var1</i> (5)	new protein	none	

**References** (1) Forst et al., 1995; (2) Givaudan et al., 1996; (3) Givaudan and Lanois, 2000; (4) Tabatabai and Forst, 1995; (5) Völgyi et al., 2000

1993; Givaudan and Lanois, 2000; Givaudan et al., 1996; Völgyi et al., 2000). In contrast, transformation of *Xenorhabdus* has not been generally successful. Although Xu et al. (1989) reported the transformation of the type strain of *X. nematophila* with a broad host-range vector, they and other workers have been unsuccessful with other strains of *X. nematophila* and other *Xenorhabdus* spp. The increase in transformation efficiency after the vector had been passaged through *X. nematophila* suggested the presence of a restriction modification system. Endonuclease activities have been detected in a range of *Xenorhabdus* spp. and it appeared that their DNA was strongly methylated (Akhurst et al., 1992). *Xenorhabdus bovienii* wild-type strains lack a functional receptor protein (LamB) in the outer membrane and as a result are unable to adsorb the phage  $\lambda$  of *E. coli*. Francis et al. (1993) developed a transposon mutagenesis system for *X. bovienii* by constitutively expressing the LamB protein on the surface of the bacterium allowing them to be infected with  $\lambda$  particles carrying the Tn10 transposon. This process produced various dye-binding, lipase, protease, hemolytic, DNase and auxotrophic mutants, i.e., alteration of phase and virulence traits. Unfortunately, use of such material for analysis of phase variation was not further continued (see Genetics/Regulation of Phase Variation in this Chapter). The Tn5 transposon was used to induce a low frequency of mutagenesis in *X. nematophila* (Xu et al., 1991) and the mini-Tn10 transposon carried on pLOF-Km<sup>r</sup> has been introduced into *X. nematophila* by conjugal transfer (Völgyi et al., 2000).

**GENES AND PROTEINS** Several genes have been identified in *Xenorhabdus* strains (Table 12). Regulation of outer membrane protein (Opns) genes of *X. nematophila* was shown in response to growth period and growth temperature (Forst

et al., 1995; Leisman et al., 1995). In *E. coli*, the OmpR and OmpC proteins form pores that allow diffusion across the outer membrane. The genes encoding them are regulated by several environmental stimuli. This is mediated by a two-component regulatory system: an EnvZ sensor protein, activated by environmental changes to phosphorylate the OmpR regulatory protein, and the *ompF* and *ompC* genes, regulated by OmpR regulatory protein. The *ompR* and *envZ* two-component signal transduction genes of *X. nematophila* were shown to constitute a single operon regulated by a  $\sigma^{70}$  promoter. One function of EnvZ in *X. nematophila* is to regulate the expression of OmpRs during the stationary period of growth.

Phase I variants of *X. nematophila* strain F1 were motile (see Physiology—swarming in this Chapter), whereas phase II variants were non-flagellated cells that did not synthesize flagellin (Givaudan et al., 1995). A locus containing two open reading frames (ORFs) was identified from phase I cells by using functional complementation of flagellin-negative *E. coli* (Givaudan et al., 1996). The sequence analysis revealed that the first ORF corresponds to the *fliC* coding for flagellin, and showed a high degree of homology between the N-terminal and C-terminal of *Xenorhabdus* FliC and flagellins from other bacteria. The second ORF, which is in the opposite orientation, encodes a homologue of the enterobacterial hook-associated protein 2, FliD. Both the *fliC* and *fliD* genes of *Xenorhabdus* were required for the complete restoration of *E. coli* motility. A sequence highly homologous to the  $\sigma^{28}$ -consensus promoter was identified upstream from the coding sequences of both genes. The structure of the *fliC* gene and its surrounding region was shown to be the same in both phase variants, but Northern blot analysis revealed that *fliC* and *fliD* were, respectively, not and weakly transcribed in phase II variants. The loci, the transcriptional activators of flagellar genes, *flhDC*, were identified (Givaudan and Lanois, 2000).

#### REGULATION OF PHASE VARIATION AND VIRULENCE

**FACTORS** Hypovirulent and avirulent mutants have been produced in *X. nematophila* by chemical and transposon mutagenesis (Dunphy, 1994; Xu et al., 1991). *N*- $\beta$ -hydroxybutanoyl homoserine lactone (HBHL), the autoinducer of the luminous system of *Vibrio harveyi*, restored virulence to previously avirulent mutants (Dunphy et al., 1997) by increasing lipase activity and lowering phenol oxidase activity in the hemolymph of insects infected with the wild strain, parameters that are associated during pathogenesis. When avirulent mutants are injected, lethality for the insect is restored upon

injection with HBHL. Because HBHL, or a closely related analogue, is excreted by the wild-type *X. nematophila*, a role for an HBHL-dependent regulatory system in its virulence process is indicated. The luminescence and virulence of *V. harveyi* against prawns (associated with a toxic extracellular protein) have been suggested to be coregulated under the control of an intercellular quorum-sensing mechanism involving the HBHL and another uncharacterized signaling molecule (Manefield et al., 2000).

Three phenotypically similar variants of *X. nematophila* strain AN6, each containing a single transposon insertion, were obtained by transposon mutagenesis (Völgyi et al., 2000). The insertions occurred at different locations in the chromosome. The variant ANV2 (AN6/1::Tn10 Amp<sup>r</sup> Km<sup>r</sup>) neither produced antibiotics nor the outer membrane protein OmpB, but produced lecithinase. The transposon had inserted in a novel gene designated *var1*, which encodes a protein of 121 amino acid residues. Complementation analysis confirmed that the pleiotropic phenotype of ANV2 was produced by inactivation of *var1*. However this *var1* gene does not complement any other phase II variants. These results indicate that inactivation of a single gene can affect multiple phase traits. More generally, all the experiments inactivating some specific phase traits by mutations or overexpression of a structural gene affect one or few functions, but none has been found to affect all the phase-related functions.

Complementation experiments showed that motility and flagellin synthesis of phase II cells cannot be recovered by placing in *trans* the *fliCD* operon from phase I cells (Givaudan et al., 1996). A gene(s) higher in the transcriptional hierarchy of the flagellar regulon, acting in *Xenorhabdus* phase II variants, was investigated. Givaudan and Lanois (2000) constructed *X. nematophila* *flhD* null mutants showing that *flhDC* operon controlled flagellin expression. But at the same time this experiment showed that lipolytic and extracellular hemolysin activities also were altered. In addition, the *flhD* null mutant displayed a slightly attenuated virulence phenotype in the insect *Spodoptera littoralis*. Thus, these data indicate that the phenotypic phase traits of *X. nematophila* (such as motility, lipase, hemolysin) participated also in the infectious process in insects. On the other hand, even if the flagellar regulon is considered to be one of the master genes involved in some aspects of phase variation, a super-regulator that controls the total phase mechanism has not been discovered. Nevertheless we should note that, in contrast with *Photobacterium*, phase variation in *Xenorhabdus* is regulated transcriptionally.



All the analyses to date have shown that different phase variant characteristics are separately controlled. However, it seems unlikely that phase variant phenotypes are all controlled independently of each other. It is probable that a master switch differentially affects a number of other regulatory systems that in turn control one or a small number of phase variant characteristics. In no case has a common regulator (controlling the whole phenomenon) been identified.

## Ecology

*Xenorhabdus* species have been found only in the intestinal tract of infective stage nematodes of the genus *Steinernema* (syn. *Neoaplectana*) and in insects killed by these nematodes. There is a high degree of specificity in these associations, with each nematode species being naturally associated with only one *Xenorhabdus* species. This specificity is determined by the ability of the nematode to retain the bacterium in its intestinal vesicle (Akhurst and Boemare, 1990; Bird and Akhurst, 1983). Although other bacteria in association with *Steinernema* have been reported (Aguillera et al., 1993; Lysenko and Weiser, 1974), closer examinations have always demonstrated that only *Xenorhabdus* have a specific association (Akhurst, 1982b; Bonifassi et al., 1999). For example, gnotobiology of *Steinernema scapterisci* and bacteriological study of its symbiont, confirmed that this nematode harbors a symbiotic species of *Xenorhabdus*, as do other *Steinernema* species. Bacteria, reported previously as associating with this nematode and belonging to several other genera, are contaminants located in the intercuticular space of the infective juveniles (Bonifassi et al., 1999). These bacteria were detrimental to nematode reproduction in *Galleria mellonella*. Combination of the symbiont and its axenic host gave the best yields of infective juveniles (IJs) when produced in this insect and in vitro production on artificial diet.

The infective stage nematodes act as vectors, transporting the bacteria into the insect via natural orifices (mouth, anus and spiracles) and then into the hemocoel (Fig. 2; see *Photorhabdus* and *Xenorhabdus* in this Chapter). The infective (dauer) nematode, a third-stage juvenile with closed mouth and anus, recommences development in the hemocoel, releasing its symbiotic bacterium and an inhibitor of the inducible antibacterial enzymes (Götz et al., 1981; Poinar and Himsworth, 1967a). As *Xenorhabdus* multiplies, it provides essential nutrients for nematode maturation and reproduction (Poinar and Thomas, 1966b) and antibiotics (Akhurst, 1982a; Li et al., 1996; Li et al., 1997; McInerney et al., 1991a; Paul et al., 1981).

*Xenorhabdus* alternate between a nutrient-rich (insect) and nutrient-poor (nematode) existence. Forst and Neilson (1996) hypothesized that phase II may be induced by the nematode gut conditions and better adapted to the nutrient-poor conditions of the intestinal vesicle of the nonfeeding nematode. However, this hypothesis does not account for the fact that the bacteria isolated from field-collected infective stage juveniles are inevitably phase I cells. Smigielski et al. (1994b) found differences in the activity of respiratory enzymes of both phase I and II cells that indicate the greater potential of phase II forms to survive in soil environments than phase I. The lack of a record of isolation of *Xenorhabdus* directly from soil may be due to its slow growth, lack of a suitable selective medium, and/or the difficulty of identifying phase II cells, which has few positive characters in most standard tests for identifying bacteria. However, Morgan et al. (1997) found that *X. nematophilus* declined very quickly in river water and soil, becoming undetectable after 2 d and 7 d, respectively. At this time, there is no satisfactory explanation of the ecological role of phase II forms.

Steinernematidae living in the soil, but specialized as insect parasites, belong to a large group of nematodes in the order Rhabditida that feed on various microorganisms. The Steinernematidae, although adapted to a particular symbiotic bacterium, have retained the ancestral microbivorous behavior allowing them to feed and reproduce on microorganisms other than their own symbiont. In spite of this feeding behavior, however, it has been shown by several researchers that bacteria other than the symbiont are not as favorable as the symbiont for growth and reproduction of *Steinernema* (Akhurst, 1982c; Boemare, 1983a; Ehlers et al., 1990), and not efficient enough to allow the perenniality of a symbiotic association (Akhurst and Boemare, 1990).

Further microbial ecological studies are needed to explain the durability over many generations of the *Xenorhabdus*–*Steinernema* intestinal symbiotic associations. The concept of the occurrence of a natural monoxeny in these bacterium–helminthic symbioses (Bonifassi et al., 1999) must be accepted, if we consider the following features. In natural conditions, if any bacterial co-associate is carried in the IJs, perhaps the IJs and associated bacteria could kill insects, but the probability of the nematode reproducing inside the insects and providing viable IJs is low. Consequently, the chances of an ecologist isolating polyxenic parasitized insects from the soil are very small due to the scarcity of these occurrences and to the short period of time between infection of an insect and its putrefaction, which would destroy any traces of the association.

## Disease

No effects on vertebrates have been demonstrated (Obendorf et al., 1983; Poinar and Thomas, 1967b; Poinar et al., 1982).

*Xenorhabdus* is an insect pathogen only when delivered into the insect hemocoel, either by their nematode symbiont or by injection; they are not pathogenic when applied per os or topically. Most are highly pathogenic for larvae of the greater wax moth, *Galleria mellonella*, with LD<sub>50</sub>s of less than 20 cells (Akhurst and Dunphy, 1993). *Xenorhabdus poinarii* has very little pathogenicity for *G. mellonella*, (LD<sub>50</sub> = 5,000 cells) when injected alone, although it is highly pathogenic when co-injected with axenic *Steinernema glaseri*, its natural host (Akhurst, 1986b). Axenic *S. scapterisci* and its symbiont *Xenorhabdus* strain UY61 alone are also not pathogenic to *G. mellonella*. The combination of both partners re-established the pathogenicity of the complex towards *G. mellonella* (Bonifassi et al., 1999). Pathogenicity of *Xenorhabdus* varies between insects, with *X. nematophila* having an LC<sub>50</sub> of about 500 for *Hyalophora cecropia* caterpillars, and no effect on maggots of the genus *Chironomus* (Götz et al., 1981). The use of a less susceptible host (e.g., *Manduca sexta*) has enabled detection of differences in pathogenicity between the two phases of a strain (Völgyi et al., 1998).

## Applications

Knowledge about the nutritional requirements provided by the symbionts would improve the industrial mass production of the entomopathogenic nematodes for biological control of insect pests. Similar comments made about *Photorhabdus* (see *Photorhabdus/Applications* in this Chapter) can be applied to *Xenorhabdus*.

Some of the secondary metabolites have commercial potential in the pharmaceutical and agro-forestry industries (Webster et al., 1998). Among the isolates examined to date from *X. nematophila* and *X. bovienii*, some indole, xenocoumarin and xenorhabdin derivatives are antibacterial, and/or antifungal, especially when they are cultivated in vitro. This is of great interest to industry (Table 10).

Moreover, the occurrence of protein toxins identified in some *Xenorhabdus* strains has enhanced interest in the bacteria alone for biological control applications. As with *Photorhabdus* (see *Photorhabdus/Applications*), programs to insert toxin genes into plant genomes for crop protection have been proposed. Today four patents have been submitted for such exploitation (East et al., 1999; Jarrett et al., 1997; Kramer et al., 1999a; and Smigielski and Akhurst, 1995).

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# The Family Vibrionaceae

J. J. FARMER III

The family Vibrionaceae contains a variety of very important organisms (Table 1). The type genus for the family is *Vibrio* and the type species is *Vibrio cholerae*, an organism that has killed millions of people during numerous devastating epidemics of cholera that terrorized most parts of the world. The family Vibrionaceae includes several species that cause intestinal tract and extraintestinal infections in both humans and animals. Many of the species of Vibrionaceae are widely distributed in the environment, where they contribute to the cycling of organic and inorganic compounds. Species of Vibrionaceae have also been widely used in physiological, biochemical, molecular biology, and pathogenicity studies (Baumann and Baumann, 1981; Baumann and Schubert, 1984; Hastings and Neelson, 1981). There are many literature citations for some of the species, but only a few for others (Table 1).

## History, Nomenclature, and Classification

### Formation of the Family in 1965

In the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957), the vibrios and related fermentative bacteria were split into a number of families (Table 2). The name Vibrionaceae was formally proposed in 1965 by Véron (Véron, 1965) as a convenient grouping for fermentative bacteria that have polar flagella and a positive oxidase reaction. One year previously, Eddy and Carpenter (1964) had also noted the similarities of *Aeromonas*, *Plesiomonas*, and *Vibrio* and thought they should not be split between the families Pseudomonadaceae and Spirillaceae (Table 2). They stated, “the creations of a new family, possibly named Vibrionaceae, might well be justified for these organisms.” This was a suggestion rather than a formal proposal, thus the name “Vibrionaceae (Eddy and Carpenter 1964)” had no standing in nomenclature. However the name Vibrionaceae was formally proposed a year later and “Vibrionaceae (Véron 1965)” does have standing in nomenclature. This classification

allowed the clear differentiation of Vibrionaceae from the closely related family Enterobacteriaceae, whose members differ in being oxidase negative and having peritrichous flagella (Table 3). Until the proposal by Véron, the genera and species of Vibrionaceae were scattered among a number of different bacterial orders and families (Table 2) (Breed et al., 1957). In this and many other early classifications, cellular morphology was of prime importance in assigning species to genera and families (Buchanan et al., 1966; Buchanan and Gibbons 1974; Campbell, 1957; Gibson et al., 1977; Hendrie et al., 1970; Shewan et al., 1960; Subcommittee, 1966, 1972). Organisms with a curved shape were often classified together as “vibrios” or “*Vibrio* species,” regardless of their other properties. This allowed anaerobic vibrios such as *Vibrio succinogenes*, microaerophilic vibrios such as *V. fetus*, and non-fermentative vibrios such as *V. psychroerythrus* to be included in the genus *Vibrio*, merely because they all had curved shapes. These and many others have now been removed from the family. Another problem with early classifications was the undue importance attached to the arrangement of flagella. Polarly flagellated and peritrichously flagellated organisms were usually placed in separate families (Table 2). It is now known that many species of Vibrionaceae have polar flagella when grown in liquid media, but synthesize peritrichous flagella when grown on solid media (Baumann and Baumann, 1981; Baumann and Schubert, 1984). For all these reasons, organisms now classified in the family Vibrionaceae were classified in different families in 1957 (Breed et al., 1957). The proposal of Véron in 1965 was very important because for the first time it brought together organisms that shared many phenotypic properties. The family name Vibrionaceae has been widely used and accepted for over 20 years (Baumann and Baumann, 1981; Baumann and Schubert, 1984; Farmer et al., 1985; Gibson et al., 1977; Hendrie et al., 1970), and there is a Subcommittee of the International Committee of Systematic Bacteriology that deals specifically with the family Vibrionaceae (Subcommittee, 1966, 1972, 1975, 1986). Table 1 gives the classification of Vibrionaceae used in this and the following two chapters and the number of literature citations found for

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This chapter was taken unchanged from the second edition.

Table 1. Nomenclature and classification of members of the family Vibrionaceae; and related organisms; guide to the literature.

	Year first described	Number of citations in: <sup>a</sup>	
		<i>Medline</i>	<i>Biosis</i>
Family Vibrionaceae (Véron 1965)			
Type genus: <i>Vibrio</i> (Pacini 1854)			
Type species: <i>Vibrio cholerae</i> (Pacini 1854)			
Type strain: ATCC 14035 (= NCTC 8021)			
Genus <i>Aeromonas</i>	1936	1,436	2,589
<i>A. hydrophila</i> group:			
<i>A. caviae</i> ( <i>A. punctata</i> )	1936	91	210
<i>A. hydrophila</i> <sup>T</sup>	1901	606	1,189
<i>A. media</i>	1983	64	78
<i>A. sobria</i>	1981	92	159
<i>A. schubertii</i>	1988	2	5
<i>A. veronii</i>	1987	4	7
<i>A. salmonicida</i>	1896	113	449
Genus <i>Photobacterium</i>	1899	333	589
<i>P. angustum</i>	1979	1	12
<i>P. leiognathi</i>	1967	51	148
<i>P. phosphoreum</i> <sup>T</sup>	1878	68	200
Genus <i>Plesiomonas</i>	1962	158	255
<i>P. shigelloides</i> <sup>T</sup>	1954	122	186
Genus <i>Vibrio</i>	1854	6,097	7,260
<i>V. aestuarianus</i>	1983	1	1
<i>V. alginolyticus</i>	1961	164	366
<i>V. anguillarum</i>	1909	101	460
<i>V. campbellii</i>	1971	3	12
<i>V. carchariae</i>	1984	1	6
<i>V. cholerae</i> <sup>T</sup>	1854	3,029	3,389
<i>V. cincinnatiensis</i>	1896	2	4
<i>V. costicola</i>	1938	27	66
<i>V. damsela</i>	1981	13	35
<i>V. diazotrophicus</i>	1982	1	5
<i>V. fischeri</i>	1896	45	190
<i>V. fluvialis</i>	1981	58	112
<i>V. furnissii</i>	1983	7	14
<i>V. gazogenes</i>	1980	3	7
<i>V. harveyi</i>	1936	78	220
<i>V. hollisae</i>	1982	13	27
<i>V. logei</i>	1980	0	5
<i>V. marinus</i>	1927	14	39
<i>V. mediterranei</i>	1986	1	2
<i>V. metschnikovii</i>	1888	16	39
<i>V. mimicus</i>	1981	33	51
<i>V. natrigens</i>	1961	2	19
<i>V. nereis</i>	1980	1	8
<i>V. nigripulchritudo</i>	1971	0	2
<i>V. ordalii</i>	1981	5	35
<i>V. orientalis</i>	1983	0	2
<i>V. parahaemolyticus</i>	1951	747	1,152
<i>V. pelagius</i>	1971	2	12
<i>V. proteolyticus</i>	1964	0	10
<i>V. salmonicida</i>	1986	4	16
<i>V. splendidus</i>	1900	1	13
<i>V. tubiashii</i>	1984	1	6
<i>V. vulnificus</i>	1979	160	284
Other organisms <sup>b</sup>			
<i>Aeromonas</i> Group 501			
Baumann's Group B2 (marine vibrio)			
Baumann's Group E3 (marine vibrio)			
NIH (Japan) biogroup 1875 (marine vibrio)			

<sup>a</sup>Citations in *Medline* from its beginning in 1966 through December 31, 1989, and in *Biosis* from its beginning in 1969 through December 31, 1989. The search was made by using both the "genus + species name" (example—*Aeromonas media*) and the "abbreviated genus name + species name" (example—*A. media*). Occasionally, this latter name resulted in erroneous citations because some species names also occur in other genera, a few of which also shared the first letter of the genus name. We have tried to remove these before making the final tabulations.

<sup>b</sup>These include strains that appear to belong in the family but lack a genus and a species name.

each genus and species. More complete information on these genera and species is given in the following two chapters.

### Classification Based on Comparisons of Nucleic Acids and Proteins

In the past, bacterial classifications were based primarily on morphological and physiological

Table 2. Classification of the Vibrionaceae and related fermentative bacteria as given in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957).

Taxon	A (possible) member of Vibrionaceae <sup>a</sup>
Class II. Schizomycetes	
Order I. Pseudomonadales	
Family IV. Pseudomonadaceae	
Genus IV. <i>Aeromonas</i>	+
Genus V. <i>Photobacterium</i>	+
Genus IX. <i>Alginomonas</i>	+?
Family VII. Spirillaceae	
Genus I. <i>Vibrio</i>	+
Order IV. Eubacteriales	
Family III. Achromobacteriaceae	
Genus IV. <i>Agarobacterium</i>	+?
Genus V. <i>Beneckea</i>	+
Family IV. Enterobacteriaceae	
Genus I. <i>Escherichia</i>	-
Genus II. <i>Aerobacter</i>	-
Genus III. <i>Klebsiella</i>	-
Genus IV. <i>Paracolonibacterium</i>	-
Genus V. <i>Alginobacter</i>	+?
Genus VI. <i>Erwinia</i>	-
Genus VII. <i>Serratia</i>	-
Genus VIII. <i>Proteus</i>	-
Genus IX. <i>Salmonella</i>	-
Genus X. <i>Shigella</i>	-

<sup>a</sup>The genera shown as "+?" probably included at least some strains that today would be considered as members of Vibrionaceae (see text).

characteristics (Breed et al., 1957; Buchanan et al., 1966; Buchanan and Gibbons, 1974). More recently, studies have focused on the structure, regulation, or function of proteins (Bang et al., 1978; reference is not an exact matchBaumann et al., 1980; Baumann and Baumann, 1973, 1978; Chludzinski et al., 1972; Cocks and Wilson, 1972; Cohen et al., 1969; Crawford, 1975; Harris and Steinman, 1977; Jensen and Rebello, 1970) and nucleic acids (Anderson and Ordal, 1972; Baumann and Baumann, 1976; Brenner et al. 1983; Gibson et al., 1979; Hori and Osawa, 1979), and have given a much better picture of the evolutionary history of Vibrionaceae and their possible origin and phylogeny.

**GUANINE PLUS CYTOSINE (GC) RATIO** One of the first techniques used to compare organisms in the family was the GC content of the DNA.

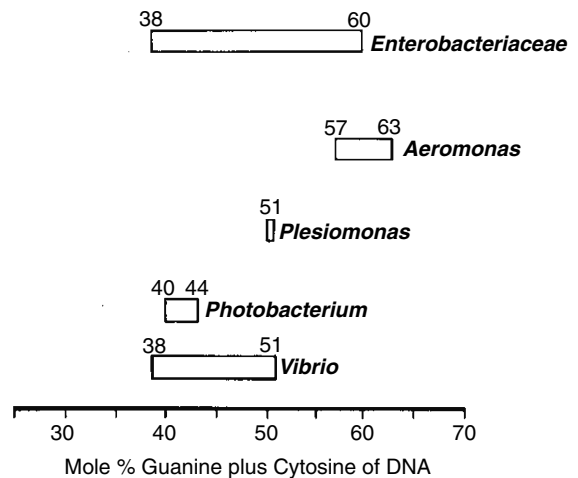


Fig. 1. The GC content of the DNA of the genera of Vibrionaceae and of the family Enterobacteriaceae.

Table 3. Characteristics of the families Enterobacteriaceae, Vibrionaceae, and Pseudomonadaceae.

Characteristic <sup>a</sup>	Property of family <sup>b</sup>		
	Enterobacteriaceae	Vibrionaceae	Pseudomonadaceae
Typical genus	<i>Escherichia</i>	<i>Vibrio</i>	<i>Pseudomonas</i>
Metabolism of D-glucose	Fermentation	Fermentation	Oxidation
Cellular morphology			
Straight rods	+ <sup>a</sup>	(-)	+
Curved rods	-	(+)	-
Flagella location	Peritrichous	Polar <sup>c</sup>	Polar
Grow in the presence of oxygen	+	+	+
Grow in the absence of oxygen	+	+	-
Oxidase test	-	+	+
Bioluminescent	-	v	-
Na <sup>+</sup> required for growth	-	(+)	-
Inhibited by the vibriostatic compound 0129	-	+	-

<sup>a</sup>These characteristics are typical of the genera and species in each family, but there are some exceptions.

<sup>b</sup>Symbols: +, most species positive; (+), many species positive; v, species-to-species variation, some species positive; (-), many species negative; -, most species negative.

<sup>c</sup>Some species of *Vibrio* have a sheathed polar flagellum when grown in a liquid medium, but have peritrichous flagella when grown on a solid medium.

**Figure 1** compares the GC content of the four genera of Vibrionaceae. The range of GC content is 38–60%, which is similar to the values found in the family Enterobacteriaceae. In the family Vibrionaceae, *Vibrio* and *Photobacterium* have a low GC content while *Aeromonas* has a high GC content.

**DNA-DNA HYBRIDIZATION STUDIES** These studies have been very helpful in defining the species in the family, and for showing that some of these species are more closely related to each other than to other organisms (Anderson and Ordal, 1972; Baumann and Baumann, 1976; Brenner et al., 1983). For example, *Vibrio cholerae* is closely related to *V. mimicus* by this technique (see The Genera *Vibrio* and *Photobacterium* in this Volume) and these two species might comprise a redefined genus *Vibrio* in a phylogenetic classification. This technique has also been used to show that *Plesiomonas shigelloides* and *Aeromonas* do not have any close relatives in *Vibrio*, *Photobacterium*, or among the species of Enterobacteriaceae (Brenner et al., 1983).

**RIBOSOMAL RNA-DNA HYBRIDIZATION; rRNA CATALOGS AND SEQUENCES** These recent studies have better defined the relationship of organisms in the families Vibrionaceae, Enterobacteriaceae, and Pasteurellaceae. The rRNA-DNA homology studies of Baumann and Baumann (1976, 1981) and the analysis of 5s rRNA catalogs of Fox et al. 1980 were based on a limited number of species (Fig. 2), but indicate that *Vibrio* and *Photobacterium* are closely related (Figs. 2 and 3). Similarly, four genera in the fam-

ily Enterobacteriaceae—*Escherichia*, *Enterobacter*, *Serratia* and *Proteus*—are closely related to each other, but not as closely related to the Vibrionaceae. *Aeromonas* and *Pasteurella multocida* occupy a position between *Vibrio-Photobacterium* and Enterobacteriaceae, but are closer to the latter. *Plesiomonas* appears to occupy a position between the families Enterobacteriaceae and Vibrionaceae.

**STRUCTURE, FUNCTION AND REGULATION OF PROTEINS** In addition to the data from nucleic acids, there are several studies that compared the regulation and activity of enzymes in different species. Enzymes from different cultures are isolated and purified and then studied in greater detail. For example, Enterobacteriaceae and Vibrionaceae have similar pathways for the synthesis of amino acids that belong to the “aspartic acid family” (Baumann and Baumann, 1973, 1981; Cohen et al., 1969). Species in both families have three isofunctional aspartokinases. One of these enzymes is feedback inhibited by the L-threonine, but the second is inhibited by L-lysine, and the third is not inhibited by L-threonine, L-lysine, or any of the aspartic acid family of amino acids (Baumann and Baumann, 1973). This pattern is different for many other bacterial families, so these data are consistent with a common evolutionary origin for Enterobacteriaceae and Vibrionaceae (Fig. 3). Similar data from studies of 3-deoxy-D-arabinoheptulose-7-phosphate synthetase (Chludzinski et al., 1972; Bang et al., 1978; Jensen and Rebello, 1970) and of enzymes of the tryptophan biosyn-

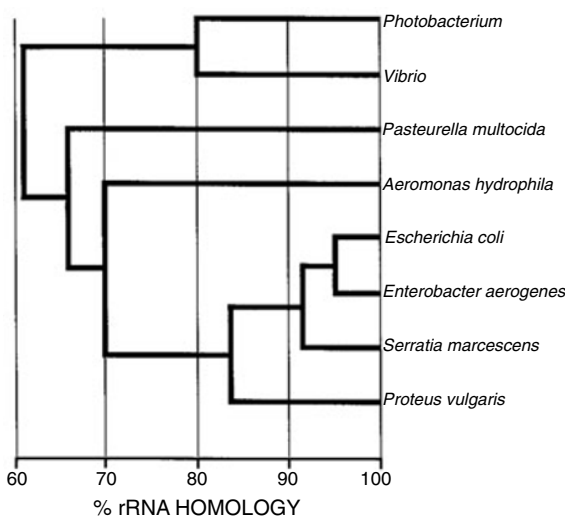


Fig. 2. Relationships of Vibrionaceae, Enterobacteriaceae, and related organisms based on rRNA-DNA hybridization. (Redrawn from the data of Baumann and Baumann, 1981; and Fox et al., 1980.)

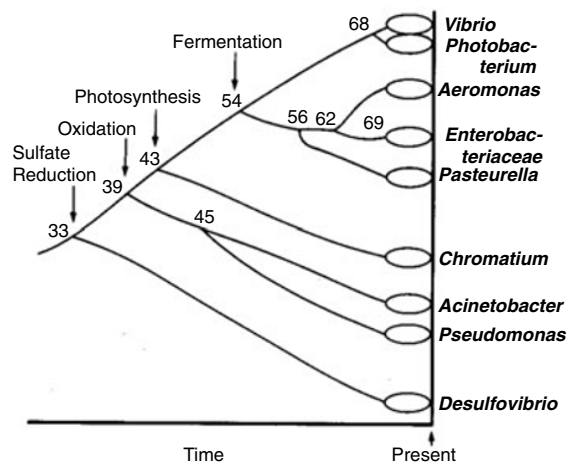


Fig. 3. Possible evolutionary pathway of the families Enterobacteriaceae, Vibrionaceae, and other members of the purple bacteria (proteobacteria) based on 16S rRNA sequence data. The two-digit numbers at the branching points are  $S_{AB}$  values, which are derived from mathematical analysis of the catalogs of oligonucleotide sequences and indicate the degree of relatedness. Redrawn from the data in Figure 4 of Fox et al., 1980.

thetic pathway (Bieger, 1978; Crawford, 1975) also give similar results. One of the few studies that has included *Plesiomonas* was by Baumann et al. (Baumann and Baumann, 1978; Baumann et al., 1980a), who compared the structure of the enzyme glutamine synthetase by microcomplement fixation. They concluded that *Plesiomonas shigelloides* was slightly more closely related to *Escherichia coli* than to *Vibrio*. This was an interesting finding since *P. shigelloides* also contains the enterobacterial common antigen (Whang et al., 1972).

### Origin and Evolution of the Family Vibrionaceae

Evidence from the primary sequence of 16S rRNA suggests that there were three major evolutionary lines in the development of life on earth, and these have been proposed as the three primary kingdoms (see Chapter 1 and Woese, 1985). All of the organisms included in the family Vibrionaceae belong to the eubacterial kingdom and to the “purple bacteria” branch of this kingdom (Woese, 1985) (Fig. 3). The purple bacteria (proteobacteria) are a diverse group that includes heterotrophic bacteria that are both fermenters and nonfermenters (Fig. 3). It also includes photosynthetic bacteria (*Chromatium*) and sulfate-reducing bacteria (*Desulfovibrio*). These data generally agree with the relatedness of the various genera, as determined by rRNA-DNA hybridization (Fig. 2).

### Removal of the Genus *Aeromonas* from the Family Vibrionaceae

Colwell et al. (1986) proposed removing the genus *Aeromonas* from the family Vibrionaceae and classifying it in a new family Aeromonadaceae as the only genus. Previous authors (Baumann and Schubert, 1984) had also commented on the divergence of *Aeromonas* from *Vibrio-Photobacterium*, which is easily visualized by the differences in the GC content of their DNA (Fig. 1). A number of studies have indicated that there is considerable divergence among the genera of Vibrionaceae and of other bacterial genera and families. However, the best way to indicate this divergence is still a controversial question. The classifications of plants, animals, and bacteria above the level of genus have been controversial and subject to numerous changes and instability with time (Mahr, 1982; Ross, 1974).

### Removal of the Genus *Plesiomonas* from the Family Vibrionaceae

MacDonell and Colwell (1985) proposed the removal of *Plesiomonas shigelloides* from the

family Vibrionaceae and its classification in the genus *Proteus* as *Proteus shigelloides*. This was based on sequence data of 5S rRNA. Other investigators had previously classified this organism in *Aeromonas* (Ewing et al., 1961) or *Vibrio* (Hendrie et al., 1971). The best place for *Plesiomonas* at a level above genus is uncertain, but all of the existing data except that of MacDonell and Colwell (1985) indicate that *Plesiomonas* is a separate well-defined genus.

Clearly, more data for all species in these families are needed, as are better definitions for the taxonomic ranks above genus (Stackebrandt et al., 1988; Wayne et al., 1987). Until these data become available and have been confirmed in several laboratories, it seems prudent to take a conservative approach and keep *Aeromonas* and *Plesiomonas* in the family Vibrionaceae because this classification has been universally accepted for many years. For those who wish to maintain this traditional classification, but still recognize the divergence of *Aeromonas*, it would be logical to reduce the family Aeromonadaceae to the level of subfamily where it would become “Aeromonadoideae subfamily nova.” The type genus for this subfamily would be *Aeromonas*, and its description would be the same as for the family Aeromonadaceae. This proposal would automatically create “Vibrionoideae, subfamily nova,” which would become the type subfamily of family Vibrionaceae. The type genus of the new subfamily Vibrionoideae would be *Vibrio*, and its description would be the same as that given by Baumann et al. (1985). Formal proposal to give these names standing in nomenclature will be made.

### Similarities and Differences Among the Genera of Vibrionaceae

As previously stated, the family Vibrionaceae was originally defined by Véron (1965) to include bacteria with the following properties: grow well on ordinary peptone media, and usually grow on media with bile salts, such as MacConkey agar; oxidase positive; motile with polar flagella. There are other similarities, but these have been the main defining characteristics of the family (Table 3). Table 4 lists the most important characteristics that differentiate the four genera in the family and that differentiate species of Vibrionaceae from species in other families. However, with the discovery of many new genera and species, it is becoming increasingly difficult to write descriptions of higher taxa such as genera and families (Stackebrandt et al., 1988; Wayne et al., 1987).

Table 4. Properties of the four genera in the family Vibrionaceae.<sup>a</sup>

Test <sup>b</sup> or property	<i>Vibrio</i>	<i>Photobacterium</i>	<i>Aeromonas</i>	<i>Plesiomonas</i>
Cause human infections	+	–	+	+
GC content of DNA (mol%)	38–51	40–44	57–63	51
Sheathed polar flagellum	+	–	–	–
Accumulate poly- $\beta$ -hydroxybutyrate, but do not utilize $\beta$ -hydroxybutyrate	–	+		
Na <sup>+</sup> required for (or stimulates) growth	+	+	–	–
Sensitive to the vibriostatic compound 0129	+	+	–	+
Lipase production	+	V	+	–
D-Mannitol fermentation	+	–	+	–

<sup>a</sup>These are properties of most species in the genus, but there are exceptions. Adapted from Baumann and Schubert (1984).

<sup>b</sup>Symbols: +, most strains (generally about 90 to 100%) positive; (+), many strains (generally about 75 to 90%) positive; V, strain-to-strain variation (generally about 25 to 75% positive); (–), many strains negative (generally about 10 to 25% positive); –, most strains negative (generally about 0 to 10% positive); ND, no data.

## Other Genera That Have Been Included in The Family Vibrionaceae

The four genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* are the well-established genera of Vibrionaceae; however, a number of other genera have been classified in the family by various authors. Two of the proposed genera (*Enhydrobacter* and *Halomonas*) contain organisms that are very different phenotypically from the four well-established genera. Inclusion of these organisms would require a drastic change in the definition of the family. Several of the genera (*Beneckea*, *Listonella*, and *Oceanomonas*) were proposed to subdivide the genus *Vibrio*. In addition, there are a number of genera, species, and unnamed organisms in the older literature that probably belong in the family, perhaps in one of the currently accepted genera or species. All of the genera are discussed alphabetically below.

### The Genus *Allomonas*

Kalina et al. (1984) isolated 24 strains of an oxidase-positive fermentative organism that had some properties of *Aeromonas* and some of *Vibrio*. The strains came from feces of healthy humans (12 strains), river water (6), and sewage (6). Kalina et al. (1984) did a numerical taxonomic analysis and defined them as “group II.” By DNA-DNA hybridization, the strains were not closely related to *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *V. cholerae*, or eight other species in the family Vibrionaceae. Based on these data, they proposed a new genus *Allomonas* in the family Vibrionaceae with a single species, *A. enterica*. However, they also mentioned that this new organism was similar in many ways to a new species in the genus *Vibrio*, *V. fluvialis*. They did not study *V. fluvialis* because

strains were not available at the time their study was done. The name *Allomonas* appeared in the literature for a brief time, but strains of *A. enterica* were later found to be identical to *V. fluvialis* by DNA hybridization and phenotypic analysis (Subcommittee, 1986). Thus, *V. fluvialis* is a senior synonym of *A. enterica*, and the latter name has not been used in the literature. Today, the genus *Allomonas* could be proposed again in an attempt to subdivide the genus *Vibrio*.

### The Genus *Beneckea*

The genus *Beneckea* was proposed as a new genus in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Campbell, 1957). It was originally classified in the Family Chromobacteriaceae (Table 2) (Breed et al., 1957). *Beneckea* included six species: *B. labra* (the type species) *B. ureasophora*, *B. chitinovora*, *B. hyperoptica*, *B. indolthetica* and *B. lipophaga*. It was defined to be a genus of rod-shaped bacteria that were Gram-negative, had peritrichous flagella, fermented carbohydrates, and digested chitin. The strains were from sea water, marine mud, brackish water, and soil. However, the genus name *Beneckea* was used very rarely until the 1970s.

Baumann et al. (1971) studied a large group of vibrios isolated in the Pacific Ocean near the Hawaiian Islands. They defined four new species of “marine vibrios” and classified them all in the genus *Beneckea*—*Beneckea campbellii*, *B. neptuna*, *B. nereida* and *B. pelagia*. They also expanded the definition of *Beneckea* to include *Vibrio alginolyticus* (*Beneckea alginolytica*) and *V. parahaemolyticus* (*Beneckea parahaemolytica*). Over the next few years, other species of *Beneckea* were described, and from 1971 until the mid-1980s the name *Beneckea* frequently appeared in the literature, often used as a synonym for “marine *Vibrio* species.” However, as additional species of *Beneckea* were described or

characterized in more detail, the genus became almost as heterogeneous as the genus *Vibrio* had been, so reference is not an exact match. Baumann et al. (1980) proposed that the genus *Beneckea* be abolished and that all the *Beneckea* species be classified in the genus *Vibrio*. The name *Beneckea* still occasionally appears in the literature, but most authors have accepted the proposal of Baumann et al. (1980b) and classify all the “marine vibrios” in the genus *Vibrio*.

### The Genus *Enhydrobacter*

The genus *Enhydrobacter* was included in the family Vibrionaceae by Staley et al. (1987) and contains a single species, *E. aerosaccus*. This organism is quite different from all other species in the family because it contains intracellular gas vacuoles (Fig. 4).

Van Ert and Staley (1971) isolated gas-vacuolated organisms from the oxygen-depleted thermocline of Wintergreen Lake, a eutrophic lake near Kalamazoo, Michigan. Water from the lake was enriched in a dilute peptone solution and then plated on media with low concentrations of organic carbon. After the plates had been incubated for a month, a colony was picked because it had a morphology typical of bacteria that form gas vacuoles. Their description of the organism included the following properties: small Gram-negative rod; nonmotile, no flagella;

intracellular gas vacuoles produced under certain conditions (Fig. 4); GC content of DNA of 66.3 mol%; poor or no growth on most rich laboratory media (generation time of 25 in a medium containing 0.1% glucose, 0.1% vitamin-free casamino acids, vitamins, and other ingredients), no sugar fermentation when done with enteric fermentation bases, weak acid production from fructose and xylose in the “OF fermentation base” of Hugh-Leifson; no growth in most media used to identify isolates in clinical microbiology laboratories; biotin and folic acid required for growth; oxidase positive; catalase positive; 21 of 42 carbon sources utilized when tested in a medium low in organic matter; indole negative; nitrate reduced only as far as nitrite; growth in both the presence and absence of oxygen (roll tube technique); growth at 25–41°C but not at 7 or 43°C; gas vacuoles produced during most growth conditions but produced better in liquid media under reduced oxygen tension. Van Ert and Staley (1971) thought that the genus *Achromobacter* was most appropriate for this unusual organism. However they later studied it by DNA-DNA hybridization (Staley et al., 1987) and found that it was 0–1% related to several species of Enterobacteriaceae, *Vibrio*, and to *Plesiomonas shigelloides*; 9% related to *Vibrio cuneatus* (a nonfermentative organisms that is no longer classified in *Vibrio*); but the highest relatedness was to *Aeromonas*. It was 11–13% related to *Aeromonas hydrophila* and 14–22% related to three other *Aeromonas* species. Relatedness to other organisms was not studied. A single strain was used to define a new genus, *Enhydrobacter*, with a new species *E. aerosaccus*, which was classified in the family Vibrionaceae (Staley et al., 1987). The type strain was designated as ATCC 27094.

No DNA-RNA hybridization or RNA sequence work has been done on *E. aerosaccus*, so its relationship to other organisms in the family Enterobacteriaceae and Vibrionaceae is uncertain. Further studies are needed to better define its closest relatives. Phenotypically, it is quite different from all the species currently included in the family Vibrionaceae.

Our laboratory examined the type strain of *E. aerosaccus* and found that it grew lightly on supplemented tryptone glucose extract (TGE) agar (van Ert and Staley, 1971), but did not grow in any of the test media used for the routine identification of Enterobacteriaceae and *Aeromonas*. It did not grow on sheep blood agar at 36°C. Phenotypically, this organism would be a strange member of the family.

### The Genus *Halomonas*

Vreeland et al. (1980) isolated nine strains of an unusual bacterium from solar condensers at the

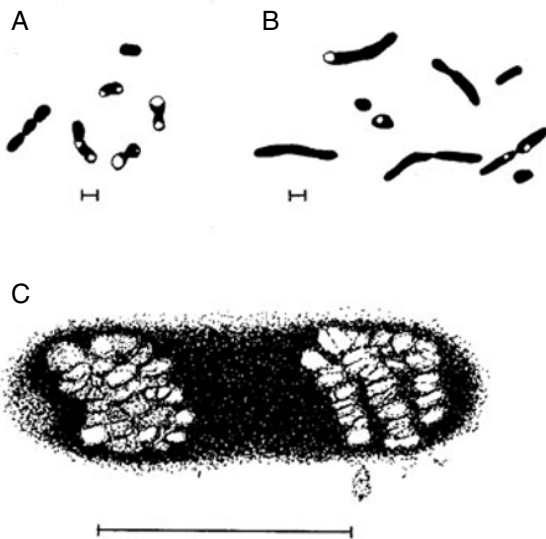


Fig. 4. Cell morphology of *E. aerosaccus* showing gas vacuoles. (A) Appearance under phase microscopy; the refractile (white) regions are the gas vacuoles. The cells were grown on Hugh-Leifson medium. (B) Same as A, except the cells were grown in tryptone-glucose extract broth plus 0.1% yeast extract. The cells are longer. (C) Electron micrograph of cells grown in low-carbon media. (All redrawn from Figures 1, 2, and 3 of van Ert and Staley, 1971.)

Antilles International Salt Company facility on Bonaire, Neatherlands Antilles (Vreeland et al., 1980). They did a number of phenotypic tests and compared their strains to other salt-requiring bacteria. Based on differences among their strains and other halophilic bacteria, they proposed a new genus, *Halomonas* (Vreeland et al., 1980). *Halomonas* had a single species, *Halomonas elongata*, and was classified in the family Vibrionaceae. The original description of *Halomonas* indicated that it fermented rather than oxidized sugars and other carbohydrates. However, it was later found that *Halomonas* is a nonfermenter rather than a fermenter (Subcommittee, 1986). Vreeland (1984) subsequently removed *Halomonas* from the family Vibrionaceae and classified it with the Gram-negative aerobic rods and cocci as a genus of uncertain affiliation (not belonging to a specified family) (see The Family Halomonadaceae in this Volume).

### The Genera *Listonella* and *Shewanella*

MacDonnell and Colwell (1985) studied the structure of 5S rRNA of many species of Vibrionaceae and other organisms and used cluster analysis to group species with similar rRNA sequences. Their data indicated that some of the species were more closely related to each other than to other organisms. Based on this criterion alone, they established two new genera in the family—*Listonella* and *Shewanella*. The proposal of *Listonella* was an attempt to subdivide the genus *Vibrio*, and the proposal of *Shewanella* was an attempt to reclassify *Pseudomonas putrefaciens* and group it with other organisms.

**THE GENUS LISTONELLA** *Listonella* was established around its type species *Listonella anguillarum* (*Vibrio anguillarum*), and included *L. damsela* (*V. damsela*) and *L. pelagia* (*V. pelagius*). It was defined almost entirely on the basis of rRNA structure and included only the following definition: “curved rods, Gram negative, motile by monotrichous or peritrichous flagella. Chemoorganotrophic, oxidase positive, associated with marine environments. Generally pathogenic for fish or eels. Do not grow at 40°C. Require NaCl for growth. GC mol% 43–46.” Interestingly, *Listonella* did not include *V. ordalii* which is highly related to *V. anguillarum* by DNA hybridization (see The Genera *Vibrio* and *Photobacterium* in this Volume) and is the only close relative based on this technique.

**THE GENUS SHEWANELLA** For many years it was known that *Pseudomonas putrefaciens* is a Gram-negative nonfermentative bacterium that can easily be isolated from the marine environment. This

organism has been classified in both *Pseudomonas* and *Alteromonas*. The proposal of reference is not an exact match MacDonnell and Colwell. (1985) classified this organism in the new genus *Shewanella*. *Shewanella* was established around its type species *Shewanella putrefaciens* (*Pseudomonas putrefaciens* = *Alteromonas putrefaciens*), and included *Shewanella hanedai* (*Alteromonas hanedai*) and a new species *Shewanella benthica*, which was also described in this paper (MacDonnell and Colwell 1985). The definition of *Shewanella* was also based almost entirely on rRNA structure, and included only the description “straight or curved rods, Gram negative, non-pigmented, motile by polar flagella. Chemoorganotrophic, oxidase positive, generally associated with aquatic or marine habitats. GC mol% 44–47.” The inclusion of *Shewanella* in the family Vibrionaceae would drastically alter the definition of the family to include nonfermentative species. Traditionally, the family has included only fermentative species.

### The Genus *Lucibacterium*

*Lucibacterium* was proposed by Hendrie et al. (1970) and was included as “Genus V” of the family Vibrionaceae in the eighth edition of *Bergey’s Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974). *Lucibacterium* contained a single species, *Lucibacterium harveyi* (*Vibrio harveyi*), and can be considered an attempt to subdivide the bioluminescent vibrios. *Lucibacterium* was used occasionally in the literature, but rarely in the last few years.

### The Genus *Oceanomonas*

*Oceanomonas* was proposed by Miyamoto et al. (1961) and classified as a new genus in the family Vibrionaceae. The type species for *Oceanomonas* was designated as *O. enteritis* (= *Vibrio parahaemolyticus*) and it also included *Oceanomonas alginolytica* (*Vibrio alginolyticus*). The main purpose of this proposal was to separate the halophilic species of *Vibrio* from *V. cholerae*, the nonhalophilic type species for the genus. The proposal of a separate genus for these two species had considerable merit because they are closely related to each other and distantly related to *V. cholerae* (see The Genera *Vibrio* and *Photobacterium* in this Volume). The grouping of these two related species into a separate genus would be the first logical step in splitting the genus *Vibrio* into phylogenetic units. However, the name *Oceanomonas* no longer has standing in nomenclature and has rarely been used in the literature.

### Bacteria that Digest Agar and Alginic Acid

In much of the older literature, bacteria that digest agar or alginic acid received special atten-



tion, and genera were set up to include all strains that digested one of these polysaccharides. The seventh edition of *Bergey's Manual of Determinative Bacteriology* (see [Table 2](#)) recognized the genera *Agarbacterium*, *Alginobacter*, and *Alginomonas*. Strains representing these genera are not readily available today, but many of them are probably species of Vibrionaceae and some are undoubtedly “marine vibrios.” The genera were very poorly defined, and one key property was the type of flagellation, either polar or peritrichous. Some genera even contained both fermentative and nonfermentative species.

**THE GENUS ALGINOBACTER** The genus *Alginobacter* was proposed by Thjøtta and Kåss (1945) and the genus name first appeared in the seventh edition of *Bergey's Manual of Determinative Bacteriology* and was classified in the family Enterobacteriaceae. The type species was *Alginobacter acidofaciens* which was the only species listed. The description included: short rods that are motile by peritrichous flagella; acid and gas produced from glucose and alginic acid; variable fermentation of other carbohydrates; citrate and alginate used as sole sources of carbon; indole negative; acetylmethyl carbinol produced (weakly); nitrate reduced to nitrite; and growth at 4, 30, 37, and 45°C.

All five strains of *A. acidofaciens* were isolated from soil. It is difficult to determine the positions of *Alginobacter* today but it appears to belong in the family Enterobacteriaceae (if the strains were oxidase negative). It is not possible to rule out the possibility that *Alginobacter acidofaciens* was a marine vibrio, since alginic acid is usually found in the marine environment.

**THE GENUS ALGINOMONAS** *Alginomonas* was proposed by Thjøtta and Kåss, (1945) and this genus was listed in the seventh edition of *Bergey's Manual* (see [Table 2](#)). The type species was *Alginomonas nonfermentans* and four other species were included—*A. terrestrialalginica*, *A. alginovora*, *A. fucicola*, and *A. alginica*. The species of *Alginomonas* were described as being “fluorescent, Gram negative, coccoid rods with one to four polar flagella, not utilizing carbohydrates, and found on algae and in sea water and soil.” The nonfermentative nature of *Alginomonas* would exclude it from the family Vibrionaceae; however, the name *Alginomonas alginica* was also used for an organism that ferments carbohydrates.

**THE GENUS ALGINOVIBRIO** The genus *Alginovibrio* was proposed by Thjøtta and Kåss (1945) for a group of alginic acid-digesting, vibrio-like bacteria isolated from sea water. No species were named, although four were

described. Formal nomenclatural proposals were made by Meland (1963), who designated *Alginovibrio aquatilis* as the type species of the genus, and named two other species, *A. norvegicus* and *A. immotus*. The species of *Alginovibrio* all were fermenters and vibrio-like, so would probably be members of Vibrionaceae. The name *Alginovibrio* has rarely been used.

**THE GENUS Agarbacterium** The genus *Agarbacterium* was proposed by Angst (1929) and it was included in the seventh edition of *Bergey's Manual of Determinative Bacteriology*. Carbohydrates were said to be “feebly attacked, if at all, some species producing acid but no gas,” so it is unclear whether strains of *Agarbacterium* were fermenters or non-fermenters. However, most strains that digest complex carbohydrates are fermenters. Other properties included: short to medium-sized rods, motile by peritrichous flagella or non-motile, pigments may be produced, and alginates may be digested. The type species was designated as *Agarbacterium aurantiacum* (Angst, 1929).

## Habitats

Species of the family Vibrionaceae occupy many different ecological niches (Baumann and Baumann, 1981; Campbell, 1957; Sakazaki and Balows, 1981; Simidu and Tsukamoto, 1985). They cause disease of humans and animals and also occur in the environment. Many factors probably govern their distribution, but four of the most important are: the particular animal or plant hosts, temperature, salinity, and depth below the surface for the species that are found in the ocean (Simidu and Tsukamoto, 1985). A few species are clearly host-adapted. *Vibrio cholerae* serogroup O1 is usually limited to humans where it causes cholera. *Aeromonas salmonicida* is adapted to salmonid fish where it causes illness and death. *Photobacterium leiognathi* is usually isolated from fish in shallow tropical water, but *P. phosphoreum* is usually found in the luminous organs of fish that live at depths of 200–1,200 (Hastings and Nealson, 1981). Until recently, strains of *P. angustum* had been isolated only from water samples taken off the coast of Hawaii. More details about the habitats of various species are given in the following two chapters.

## Isolation

Many of the species of Vibrionaceae are easy to isolate because they grow well on the ordinary peptone-type media used in general and clinical microbiology (Baumann and Baumann, 1981). [Table 5](#) lists some of the general and selective

Table 5. The usefulness of common media for the isolation of the four genera of the Vibrionaceae.<sup>a</sup>

Medium	Usefulness for: <sup>b</sup>			
	<i>Vibrio</i>	<i>Photobacterium</i>	<i>Aeromonas</i>	<i>Plesiomonas</i>
Plating media often used in clinical microbiology laboratories				
Sheep blood agar	++++	++	++++	++++
MacConkey agar	++		++++	++++
Alkaline peptone water	++++	++	+++	+++
Nonselective marine media				
Marine broth	++++	++++	++	++
Marine agar	++++	++++	++	++
Photobacterium broth	++++	++++	++	++
Photobacterium agar	++++	++++	++	++
Selective media for <i>Aeromonas</i> and <i>Plesiomonas</i>				
Bile salts-brilliant green agar			++++	++++
Dextrin-fuchsin-sulfite medium			++++	
Rimler-Shotts medium			++++	
<i>Aeromonas</i> agar			++++	
Blood agar plus ampicillin			++++	
DNase agar plus ampicillin and toluidine blue			++++	
MacConkey agar plus ampicillin and Tween 80			++++	
Pril-xylose-ampicillin agar			++++	
Starch-ampicillin agar			++++	
Selective <i>Vibrio</i> media <sup>c</sup>				
TCBS agar	++++	++	+	-
<i>Vibrio</i> agar	++++			

<sup>a</sup>See The Genera *Vibrio* and *Photobacterium* and The Genera *Aeromonas* and *Plesiomonas* in this Volume for more details.

<sup>b</sup>The symbols “+” to “++++” and “-” indicate the general usefulness of the medium and apply to most species in the genus; however, some species and strains may be exceptional and may differ on a particular medium. For example, most strains of *Vibrio hollisae* do not grow well on TCBS agar (see The Genera *Vibrio* and *Photobacterium* and The Genera *Aeromonas* and *Plesiomonas* in this Volume). A blank space indicates that data are not available or are contradictory.

<sup>c</sup>Most of the media formulated for *Vibrio* isolation were specifically designed to isolate *V. cholerae*.

media, which are discussed in more detail in The Genera *Vibrio* and *Photobacterium* and The Genera *Aeromonas* and *Plesiomonas* in this Volume. Most strains have been isolated from human or animal clinical specimens, fresh water, or salt water close to the shore. Collecting water samples can be done with a number of commercial sampling devices, or simply with a sterile container tied to a string.

Samples are collected and then plated (in the field or in the laboratory) onto solid media and/or placed in liquid enrichments. The incubation temperature and NaCl content of the medium are two important variables to consider in isolation. A few species are clearly psychrophilic and do not even grow at 25°C. Other species grow well at 25°C but not at 35–37°C, the temperature commonly used in clinical laboratories. Most species of Vibrionaceae grow in commercial peptone media that contain 0.5 to 0.85% NaCl in their formulas, but a few require a higher concentration for growth.

## Identification

The first and most important step in identification is to determine that a clinical or environ-

mental isolate is probably a member of the family Vibrionaceae. Tables 3 and 4 list some of the properties that are suggestive. The next step is usually to identify the isolate to the species level by a variety of phenotypic tests that are described in The Genera *Vibrio* and *Photobacterium* and The Genera *Aeromonas* and *Plesiomonas* in this Volume.

## Human Clinical Isolates

About half the species of Vibrionaceae occur in human clinical specimens, and their identification is not difficult. Strains of Vibrionaceae are rare, however, compared to Enterobacteriaceae, so laboratory personnel may not be as familiar with them. Commercial identification systems have not been as successful in identifying Vibrionaceae as they have been with Enterobacteriaceae (Farmer et al., 1985). However, many manufacturers have now obtained strains of Vibrionaceae and improved the accuracy of their product. Identification of Vibrionaceae with these commercial kits will likely continue to improve with time. *Vibrio cholerae* O1, *V. cholerae* non-O1, *V. parahaemolyticus*, *V. alginolyticus* and the *Aeromonas hydrophila* group are the most common clinical isolates. Identification of

these species is not difficult, regardless of the method used.

### Environmental Isolates

Identification of environmental isolates can be much more difficult because this group has not been studied as well as the clinical group. Identification of *Aeromonas* and *Plesiomonas* strains is not difficult, and can be done with the same media and methods used for clinical isolates. Identification of *Vibrio* and *Photobacterium* isolates is more difficult and requires special media and methods because of their special temperature and ionic requirements.

### Computer Identification

Because many of the species of Vibrionaceae are similar in their phenotypic properties, several computer programs have been developed in our laboratory for the routine identification of *Aeromonas*, *Plesiomonas*, *Vibrio*, and *Photobacterium* isolates. The programs are based on mathematical analysis of the results of 45 to 50 simple biochemical tests. The test media are inoculated and read at one and two days (and up to seven days if desired). Each test is coded as "+" or "-" for the computer, which then compares the profile of the unknown strain with all the organisms in the data base. The approach is based on the normalized likelihood calculation described by LaPage (1974). Two different programs are used: The first, named "George," is used for all the species of Enterobacteriaceae and for the species of Vibrionaceae that grow well at 35–37°C in media with no added salt (0.5–1.0% NaCl). A second program, named "Neptune," was designed specifically to identify the halophilic species of *Vibrio* and *Photobacterium*. The biochemical tests used in this program have marine cations added to give a higher content of Na<sup>+</sup>, K<sup>+</sup>, and Mg<sup>++</sup>, and all incubations are done at 25°C since many of these species do not grow well at 35–37°C. Originally these computer programs were written in FORTRAN computer language and run on a mainframe computer. However, because many potential users did not have access to a mainframe computer, the programs were rewritten in BASIC computer language for microcomputers that are IBM compatible and have the DOS operating system. Interested persons should write to J. J. Farmer to obtain further information for obtaining these. We find that both programs are extremely useful for comparing a clinical or environmental isolate with a large data base of species, biogroups, and individual strains.

Details on the characteristics of the various genera of the Vibrionaceae are given in Chapters 157 and 158.

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# The Genera *Vibrio* and *Photobacterium*

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## Introduction

Both *Vibrio* and *Photobacterium* are old genera that were described in the 1800s. The genus name *Vibrio* was coined by Pacini in 1854 during his studies on cholera, and it is one of the oldest names for a bacterial genus. Pacini also named the cholera bacillus, which eventually became *V. cholerae*, the type species for the genus *Vibrio*. Much of the history and literature on *Vibrio* came from medical microbiology and concerned cholera and other similar vibrios that early bacteriologists had difficulty in differentiating from the true cholera bacillus. In contrast, the genus name *Photobacterium* was coined by Beijerinck (188a) who was interested in environmental microbiology. Most of the literature on *Photobacterium* and on the marine species of *Vibrio* has come from environmental microbiologists, particularly those studying bacteria found in the sea. Unfortunately, there has been a poor interchange of ideas, information, and bacterial cultures between these two disciplines, which has led to considerable confusion in the literature. The methods for isolation and identification used by the two groups of workers are quite different and add further confusion. Several of the *Vibrio* species are very important in human or animal disease, and many of the species of *Vibrio* and *Photobacterium* are widely distributed in the environment. Many papers review various aspects of the two genera including: animal diseases (Anderson and Conroy, 1970; Baross et al., 1978; Sinderman, 1970); basic biology (Baumann and Baumann, 1977); enzyme structure and function (Bang et al., 1978b; Baumann and Baumann, 1973, 1978; Crawford, 1975; Gee et al., 1975); cellular structure or composition (Eberhard and Rouser, 1971); ecology and distribution (Golten and Scheffers, 1975; Harrell et al., 1976); human infections (Blake et al., 1979; Ryan, 1976; Wachsmuth et al., 1980; World Health Organization Scientific Working Group, 1980); physiology and metabolism (Doudoroff, 1942b; Eagon and Wang, 1962; Gauthier, 1976; Humm, 1946; Ingra-

ham, 1962; Payne et al., 1961; Richter, 1928; Stanier, 1941); nucleic acid structure, function, or relatedness (Baumann and Baumann, 1976; Schiewe et al., 1977); nutrition (Doudoroff, 1942a); symbiotic relationships (Reichelt et al., 1977; Ruby and Morin, 1978); and taxonomy (Baumann et al., 1971a; Baumann et al., 1984; Fitzgerald, 1977; Hendrie et al., 1970; Reichelt and Baumann, 1975; Shewan and Véron, 1974; Singleton and Skerman, 1973).

## History

The Concept of “Marine Vibrios”;  
Requirement for Na<sup>+</sup> and Other Ions  
(Baumann and Baumann, 1981)

The first comprehensive study of bacteria indigenous to the oceans was done by Bernhard Fischer (1894) of the University of Kiel. His major conclusions have been confirmed and extended by numerous investigators who have shown that the majority of the heterotrophic bacteria in the open oceans are Gram-negative, straight or curved rods or spirals that are usually motile by means of flagella (Baumann et al., 1971a, 1972; MacLeod, 1965, 1968; Pfister and Burkholder, 1965; Sieburth, 1979). Most of these organisms are eubacteria and have rigid cell walls. Fischer (1894) made the important observation that the highest viable counts (plate counting method) were obtained when seawater or 3% NaCl was included in the nutrient medium. This finding was subsequently interpreted to be primarily an osmotic phenomenon, since many marine bacteria were found to lyse in dilute media (Harvey, 1915; Pratt, 1974). However, Richter (1928) clearly demonstrated a specific Na<sup>+</sup> requirement for the growth of a marine luminous strain and, in addition, showed that complex media were not suitable for establishing this requirement since they contain substantial levels of inorganic ions (for reviews dealing with the early literature, see Larsen, 1962; MacLeod, 1965, 1968). Since these results were overlooked, and complex media continued to be used, considerable controversy persisted concerning the presence and stability

of this character. MacLeod independently reestablished the early findings of Richter and developed synthetic media for testing the presence of a  $\text{Na}^+$  requirement (MacLeod, 1968). The extensive application of these methods has since established that all or most Gram-negative marine bacteria have a specific requirement for  $\text{Na}^+$  (Baumann et al., 1971a, 1971b, 1972, 1973; Hidaka and Sakai, 1968; MacLeod, 1965, 1968). Furthermore, the work of MacLeod (1968) and others (reviewed by Pratt, 1974) has indicated the genetic stability of this requirement. Using 31 different marine isolates, including seven species of facultative anaerobes (with GC contents of 39–48 mol%), 12 species of nonfermentative marine organisms (with GC contents in their DNAs of 30–68 mol%), and one strain of a marine host-independent bellovibrio, Reichelt and Baumann (1974) studied the effect of NaCl concentration on growth rate and cell yield in media containing 50 mM  $\text{Mg}^{++}$  and 10 mM  $\text{Ca}^{++}$  (marine medium) and 2 mM  $\text{Mg}^{++}$  and 0.55 mM  $\text{Ca}^{++}$  (terrestrial medium). The optimum growth rates and cell yields in the marine medium ranged from 70 to 300 mM NaCl, while the optima in the terrestrial medium ranged from 100 to 460 mM. In many strains, the higher concentrations of  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  present in the marine medium reduced the amount of NaCl required for optimal growth rate and yield and decreased the generation time. Some strains did not grow unless the medium contained the higher  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  concentrations, indicating that the addition of 3% NaCl to a terrestrial medium will not make it suitable for the cultivation of many common marine bacteria. The extent to which the  $\text{Na}^+$  requirement may be partially reduced by other ions differs considerably among marine isolates (MacLeod, 1965, 1968; Pratt, 1974). In the case of *Alteromonas haloplanktis* (the organism used in the extensive studies of MacLeod and his collaborators),  $\text{Li}^+$  has little or no sparing effect, while in the case of *Vibrio parahaemolyticus*, this ion is able to considerably reduce the concentration of  $\text{Na}^+$  required (Morishita and Takada, 1976).

Detailed studies of the physiological basis of the  $\text{Na}^+$  requirement have so far been restricted to a strain of the species *Alteromonas haloplanktis*. In this organism  $\text{Na}^+$  is essential for: 1) the function of all the examined permease systems which include those involved in the uptake of amino acids, tricarboxylic acid cycle intermediates, galactose, orthophosphate, and  $\text{K}^+$  (Thompson and MacLeod, 1973); and 2) maintenance of the integrity of the cell wall (Forsberg et al., 1970). Conclusion (1) has been extended to the marine species *Vibrio fischeri* (Drapeau et al., 1966) and to *Pseudomonas doudoroffii* which requires 75mM  $\text{Na}^+$  for the optimal rate of

uptake of D-fructose (Baumann and Baumann, 1981). Conclusion (2) has also been extended to an *Alteromonas espejiana* strain, the host of the lipid-containing, marine bacteriophage PM-2 (Baumann and Baumann, 1981). In a recent survey of the effect of  $\text{Na}^+$  on the integrity of the cell wall, it was found that in the absence of this ion a weakening of the outer membrane occurred in 14 of 20 marine strains examined. These observations suggest that the requirement for  $\text{Na}^+$  by marine bacteria is a complex, multi-genetic trait which would not be readily lost by mutation. In contrast to the marine bacteria and the extreme halophiles which require over 3 M  $\text{Na}^+$  (Larsen, 1962), the growth of most Gram-negative terrestrial organisms does not appear to be  $\text{Na}^+$  dependent. Where a requirement has been demonstrated it has generally been found to be considerably lower than that observed in marine bacteria and may only be present under certain conditions of cultivation (Kodama and Tanaguchi, 1976; Reichelt and Baumann, 1974). An interesting exception are the rumen bacteria, which live in an environment having a relatively high concentration of this ion (Caldwell and Hudson, 1974; Reichelt and Baumann, 1974). The level of the  $\text{Na}^+$  requirement and its stability imply that marine bacteria would not be able to colonize most terrestrial habitats. Conversely, there is considerable evidence that Gram-negative terrestrial bacteria do not survive in the marine environment (Jannasch, 1968; Moebus, 1972). These observations suggest an ecological separation of Gram-negative marine and terrestrial organisms as a consequence of specific adaptations to their respective habitats. In the case of marine bacteria, this attribute was probably acquired as a result of physiological adaptations to life in an environment having a relatively constant ionic composition. In this context, it is curious that many marine bacteria appear to grow better at 50–75% seawater than at 100% concentration (Gundersen, 1976) and that the optimal concentration of  $\text{Na}^+$  (70–300 mM) for the growth of a number of marine isolates is considerably lower than the  $\text{Na}^+$  concentration in seawater (450–480 mM) (Reichelt and Baumann, 1974). No obvious correlation has been observed between the source of isolation of the strains and the amount of  $\text{Na}^+$  necessary for optimal growth.

Another fundamental question is whether there are bacterial species unique to the marine environment. Stanier (1941) succinctly formulated this problem and proposed an experimental solution, the essence of which was a comparison of the bacterial flora with similar biological functions (e.g., mineralization of simple organic compounds) in both marine and terrestrial habitats. The application of this approach has been facilitated by the existence of relatively

specific enrichment methods for the isolation of terrestrial pseudomonads as well as by the extensive phenotypic characterization of these organisms, which allows the ready identification of species (Stanier et al., 1966). In utilizing enrichment methods for the isolation of marine bacteria, it is essential to use seawater samples obtained aseptically at locations where contamination by terrestrial organisms is minimal or absent. Using these precautions, it could readily be demonstrated that when a marine inoculum was used in enrichment cultures selective for certain species of terrestrial pseudomonads, the resulting flora consisted of facultative anaerobes and nonfermentative organisms which were different from terrestrial species (Baumann et al., 1971a, 1971b, 1972, 1973; Reichelt and Baumann, 1973). These results established that the mineralization of simple organic compounds in the ocean is done by a bacterial flora that is different from those with this role in terrestrial habitats. This may not apply to estuarine habitats, since these environments are complex with respect to the diversity of habitats, salinity, availability of nutrients, and contamination by terrestrial organisms. The designation of a species as "estuarine" has no real conceptual meaning since it is not yet known whether there are any species indigenous to estuaries or other coastal habitats. Since the open oceans contain relatively low concentrations of bacteria relative to those found directly off shore, the inability to detect a particular species in the open ocean may simply be due to the limitation of sample size.

About 50% of the oceans (by area) reach depths where the pressures range from 380 to 1,100 atmospheres (ZoBell, 1963). Consequently, an important question has been the possible existence of bacteria which are specifically adapted to life at high hydrostatic pressures and which would be inhibited or killed by exposure to one atmosphere. The resolution of this problem has been undertaken by H. Jannasch and his collaborators who have constructed special samplers which allow the cultivation of marine microorganisms at the hydrostatic pressures at which they were sampled without introducing any decompression steps (Jannasch and Wirsen, 1977; Jannasch et al., 1976). Although detailed investigations of this problem are few, the initial results strongly suggest that if microorganisms specifically adapted to high pressures do exist, their numbers constitute a relatively small proportion of the total metabolically active, heterotrophic, bacterial flora of the deep oceans.

Over 90% of the marine environment (by volume) has a temperature below 5°C (ZoBell, 1963). Consequently, it is also of considerable interest to know whether the psychrophilic isolates from the ocean differ from previously char-

acterized mesophiles only in their relation to temperature or whether they actually constitute different species. An answer to this question should be readily obtained by application of the various methods used for the characterization of marine bacteria.

One practical consequence of the marine nature of most of the species of *Vibrio* and *Photobacterium* is shown in Table 1, which indicates that biochemical test results used for routine identification vary depending on the Na<sup>+</sup> content of the media. The addition of NaCl usually results in an increase in the number of positive reactions and in the rapidity of the positive results.

### History—Medical Aspects

The cholera bacillus, *Vibrio cholerae*, was first cultured by Koch in 1882 during an epidemic of cholera in India and Egypt. This led to an intensive study of the vibrios by many workers with the main goal of differentiating the "nonpathogenic" environmental and marine vibrios from the true cholera bacillus. No other pathogenic species of *Vibrio* were documented until 1950, when Fujino isolated *V. parahaemolyticus* from an outbreak of severe food poisoning in Osaka, Japan (see Fujino et al., 1974). Recently, a number of other species have been described and their role in causing human disease has been postulated or shown.

### Nomenclature and Classification

In the last two decades the vibrios and photobacteria have changed from a poorly characterized heterogeneous group of organisms to several well-understood natural groups. This has been due to the transfer of "nonfermentative vibrios," "aerobic vibrios," and "microaerophilic vibrios" to other genera such as *Campylobacter* (*Vibrio fetus*), *Wolinella* (*Vibrio succinogenes*), *Pseudomonas*, and *Alteromonas* (Baumann and Baumann, 1981). The species currently recognized in *Vibrio* and *Photobacterium* are listed in Table 2. The genera *Vibrio* and *Photobacterium* are classified in the family Vibrionaceae along with two other genera, *Aeromonas* and *Plesiomonas*. Fig. 1 in Chapter 156 gives the gc content of the genera of vibrionaceae. It can be used as a first step in determining the relatedness of genera. *Vibrio* is closely related to *Photobacterium*, which is expected because of their phenotypic similarities, but these genera are more distantly related to *Aeromonas* and to the family enterobacteriaceae (Fox et al., 1980). These relationships were shown by nucleic acid hybridization experiments that are summarized in Fig. 1



Table 1. The effect of media (especially Na+ content) and test conditions on biochemical test results for some species of the genus *Vibrio*.

Test or property	Percentage positive for: <sup>a</sup>									
	<i>V. cholerae</i>	<i>V. mimicus</i>	<i>V. metschnikovii</i>	<i>V. hollisae</i>	<i>V. damsela</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>V. alginolyticus</i>	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>
Indole production										
Peptone water	86	94	22	30	0	0	0	14	35	39
Peptone water + 1% NaCl	86	98	26	93	0	0	0	24	80	65
Heart infusion + 1% NaCl	97	95	17	97	0	13	11	42	89	94
Methyl red										
Standard	25	14	17	NG	NG	0	0	NG	NG	NG
Standard + 1% NaCl	99	99	96	0	100	96	100	77	78	79
Voges-Proskauer										
Standard	74	2	26	NG	NG	0	0	NG	NG	NG
Standard + 1% NaCl	73	1	24	0	33	0	0	8	0	0
Standard + 1% NaCl; Barrit method	93	9	96	0	95	0	0	83	0	0
Arginine dihydrolase										
Moeller's	0	0	0	0	81	52	0	0	0	0
Moeller's + 1% NaCl	0	0	59	0	95	93	100	0	0	0
Lysine decarboxylase										
Moeller's	99+	99	13	0	0	0	0	29	63	85
Moeller's + 1% NaCl	99+	100	36	0	52	0	0	99	100	99
Ornithine decarboxylase										
Moeller's	99+	99	0	0	0	0	0	3	71	47
Moeller's + 1% NaCl	99+	99	0	0	0	0	0	53	89	53
Gelatin hydrolysis										
Standard	52	60	65	0	5	41	56	45	66	55
Standard + 1% NaCl	62	63	38	0	6	85	86	76	89	75
Esculin hydrolysis										
Standard	0	0	4	0	0	0	0	0	0	0
Standard + 1% NaCl	2	0	59	0	0	8	0	2	1	39
Nitrate→Nitrite										
Standard	99+	99+	0	NG	NG	59	33	NG	0	2
Standard + 1% NaCl	99+	100	0	100	100	100	100	100	100	100

<sup>a</sup>The number indicates the percentage positive after 48 h of incubation at 36°C; NG, most cultures do not grow in the medium.

Table 2. The species of *Vibrio* and *Photobacterium* and their location in human clinical specimens.

	Occurrence in human clinical specimens: <sup>a</sup>	
	Intestinal	Extraintestinal
<i>Vibrio alginolyticus</i>	+	++
<i>V. carchariae</i>	-	+
<i>V. cholerae</i>		
Serogroup O1	++++	+
Serogroup non;nNO1	++	++
<i>V. cincinnatiensis</i>	-	++
<i>V. damsela</i>	-	++
<i>V. fluvialis</i>	++	-
<i>V. furnissii</i>	++	-
<i>V. hollisae</i>	++	-
<i>V. metschnikovii</i>	-	+
<i>V. mimicus</i>	++	+
<i>V. parahaemolyticus</i>	++++	+
<i>V. vulnificus</i>	+	+++
<b>Species that do not occur in human clinical specimens<sup>b</sup></b>		
Genus <i>Vibrio</i>	<i>V. marinus</i>	<i>V. proteolyticus</i>
<i>V. aesturianus</i>	<i>V. mediterranei</i>	<i>V. salmonicida</i>
<i>V. anguillarum</i>	<i>V. natriegens</i>	<i>V. splendidus</i>
<i>V. campbellii</i>	<i>V. nereis</i>	<i>V. tubiashii</i>
<i>V. costicola</i>	<i>V. nigripulchritudo</i>	Genus
<i>V. diazotrophicus</i>		<i>Photobacterium</i>
<i>V. fischeri</i>	<i>V. ordalii</i>	<i>P. angustum</i>
<i>V. gazogenes</i>	<i>V. orientalis</i>	<i>P. leiognathi</i>
<i>V. harveyi</i>	<i>V. pelagius</i>	<i>P. phosphoreum</i>
<i>V. logei</i>	<i>V. orientalis</i>	

<sup>a</sup>The symbols +, ++, +++, and +++++ give the relative frequency of each organism in specimens.

<sup>b</sup>Theoretically any of these species could be found in feces after the ingestion of seafood or water that contains them. Based on published data, this must be a rare event.

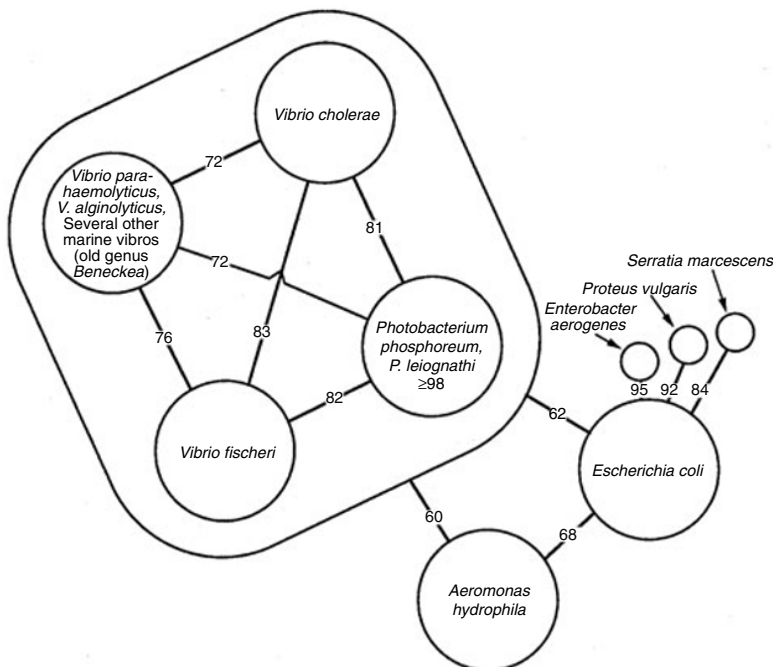
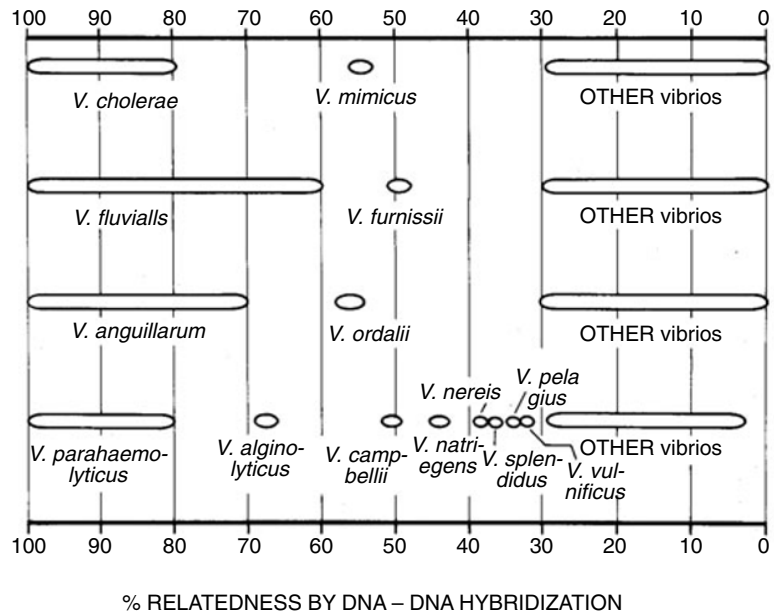


Fig. 1. Relatedness of the species of *Vibrio* and *Photobacterium* to each other and to other organisms in the families Enterobacteriaceae and Vibrionaceae based on the DNA-RNA hybridization studies of Baumann and Baumann and their coworkers. Numerical values represent average percent homologies. (Redrawn from Baumann and Baumann, 1981.)

Fig. 2. Related species in the genus *Vibrio* based on DNA-DNA hybridization. (Redrawn from our own data that was summarized by reference is not an exact match Brenner et al., 1983.)



(Baumann and Baumann, 1981). More distant relatives include the purple photosynthetic bacterium *Chromatium* and the nonfermentative genera *Pseudomonas* and *Acinetobacter* (see Fig. 3 in The Family Vibrionaceae in this Volume). Also Table 4 in The Family Vibrionaceae in this Volume lists some properties that differentiate the four genera in the family Vibrionaceae. Most of the *Vibrio* species are not closely related to each other in a phylogenetic sense (Baumann and Baumann, 1981; Baumann and Schubert, 1984; Brenner et al., 1983a). Thus, *Vibrio* is a heterogeneous genus, in a manner similar to the genus *Pseudomonas*. This is clear from DNA-DNA hybridization experiments that have been confirmed in several laboratories (Anderson and Ordal, 1975) and are shown in Figs. 2 and 3. Since *V. cholerae* is the type species for the genus, the genus definition must be built around it. *V. cholerae* is closely related to *V. mimicus*, but not closely related to the other vibrios (Fig. 2). However, another major evolutionary line includes several of the wellknown *Vibrio* species: *V. parahaemolyticus* is closely related to *V. alginolyticus* and greater than 30% related to several other species (Figs. 2 and 3). Most of the other species do not have close relatives, but there are a few exceptions: *V. fluvialis* with *V. furnissii*, *V. anguillarum* with *V. ordalii*, *V. splendidus* biogroup 1 with *V. splendidus* biogroup 2, *V. pelagius* biogroup 1 with *V. pelagius* biogroup 2, and *P. leiognathi* with *P. angustum* (Fig. 3). Even within a well-defined species there can be considerable divergence. For example, *V. gazogenes* contains three to four DNA hybridization groups (Fig. 4) (Farmer et al., 1988) that are almost identical in their phenotypic properties. Similar genetic com-

parisons based on a large number of strains have been done for only a few *Vibrio* species. The conclusions for relatedness in *Vibrio* based on nucleic acid hybridization generally agree with those based on protein structure (see Fig. 5.20 of Baumann et al., 1984). However, recent data based on RNA sequences have not been in agreement, and have led to some proposals that need to be confirmed in other laboratories and with different methods before they can be seriously considered.

## Habitats

The species of *Vibrio* and *Photobacterium* are widely distributed in the marine environment, and a few species infect marine animals, particularly if they are stressed. In addition, 12 of the *Vibrio* species cause intestinal or extraintestinal human infections (Blake et al., 1979; Colwell, 1984) or have been isolated from human clinical specimens. *Photobacterium* species have been isolated from human clinical specimens.

### Infections of the Intestinal Tract—Cholera and Other Diarrheas

Five of the *Vibrio* species appear to cause diarrhea in humans. *V. cholerae* is well known as the cause of cholera (Barua, 1970; Finkelstein, 1973; Pollitzer, 1959), which is a distinct clinical entity in its most severe form, but in its milder form may be hard to distinguish from other watery diarrheas. *V. parahaemolyticus* is a well-documented cause of acute gastroenteritis (Fujino et al., 1974; Miwatani and Takeda, 1976). More recently, *V.*

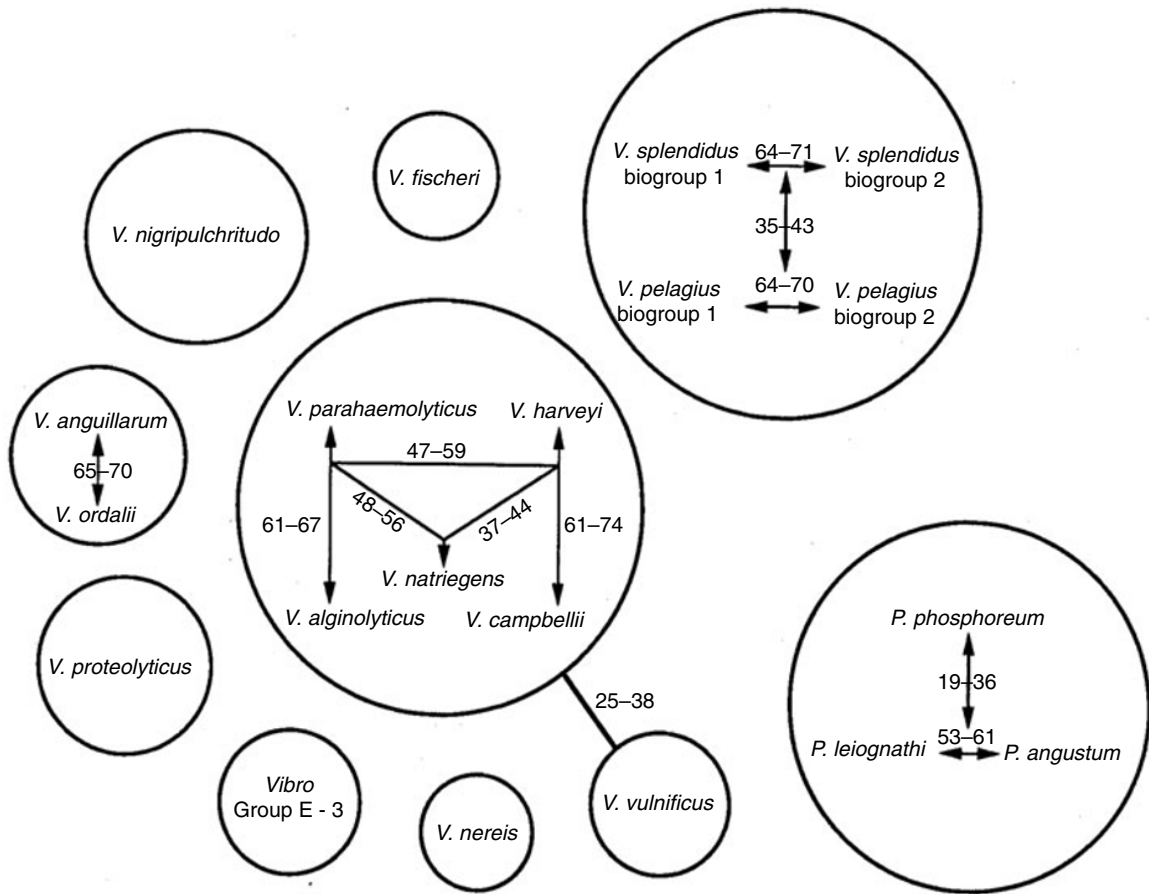


Fig. 3. Related species in the genera *Vibrio* and *Photobacterium* based on DNA-DNA hybridization; strains within each species and biogroup are related by DNA homologies of over 80%. Numbers indicate the range of DNA homology values between different organisms. Circles that are not connected are related by 30% or less (circle size has no meaning). (Redrawn from Fig. 44 of Baumann and Baumann, 1981.)

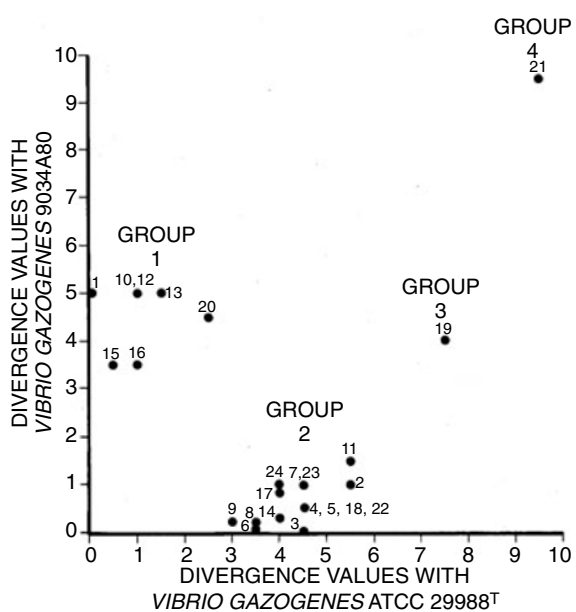


Fig. 4. Subgroups in the species *Vibrio gazogenes* based on divergence values in DNA-DNA hybridization experiments (these values reflect unmatched DNA sequences).

*fluviialis* (Huq et al., 1980; Lee et al., 1981), *V. hollisae* (Hickman et al., 1982; Morris et al., 1982), and *V. mimicus* (Davis et al., 1981) have been implicated as causes of diarrhea. *V. furnissii* (Brenner et al., 1983b) has been isolated from a few individuals with diarrhea, but there is no evidence that it can actually cause diarrhea.

**Extraintestinal Infections**

Strains of *Vibrio* have been isolated frequently from certain extraintestinal infections but are rarely isolated from others. Table 3 summarizes the sources of cultures studied at the *Vibrio* Reference Laboratory, Centers for Disease Control (CDC) Atlanta, Georgia. *Vibrio* species are often isolated from blood, arm and leg wounds, infected eyes and ears, and from gallbladders removed at surgery. They are rarely reported from patients with meningitis or pneumonia or from infections of the reproductive organs or urinary tract (Tison and Kelly, 1984). Within the genus *Vibrio* there is a division among the species which cause intestinal infection and those

Table 3. Sources of *Vibrio* isolates.

	<i>V. cholerae</i>		<i>V. mimicus</i>	<i>V. metschnikovii</i>	<i>V. cincinnatiensis</i>	<i>V. holtsiae</i>	<i>V. damsela</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>V. alginolyticus</i>	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. carchariae</i>
	O1	Non-O1											
Human													
Feces or intestine	118	94	39	0	2	30	0	15	16	4	114	6	0
Spinal fluid	0	1	0	0	1	0	0	0	0	0	0	2	0
Blood	1	42	0	1	0	2	0	0	0	0	2	63	0
Wound													
Hand or arm	0	2	0	0	0	0	1	0	0	1	3	12	0
Foot or leg	1	9	1	0	1	0	9	0	0	12	7	10	1
Other or unknown	1	3	1	0	0	0	0	0	1	9	1	1	0
Ear	0	24	6	0	1	0	0	0	0	18	0	0	0
Eye	0	0	0	0	0	0	0	0	0	2	0	0	0
Gall bladder	1	1	1	0	0	0	0	0	0	1	0	0	0
Urine	0	4	0	2	0	0	0	0	0	0	0	1	0
Respiratory tract	0	10	0	0	0	0	0	0	0	6	0	1	0
Other or unknown	4	12	5	4	1	1	0	0	0	6	0	8	0
Nonhuman													
Animals, nonmarine													
Primate	0	1	0	0	0	0	0	0	0	0	0	0	0
Pet or farm	0	6	0	0	4	0	0	0	1	0	0	0	0
Other	0	1	0	0	2	0	1	0	1	0	0	0	0
Animals, marine													
Fish	0	7	0	0	0	0	7	0	0	2	1	0	0
Oyster	8	29	9	0	0	0	1	5	0	0	11	4	0
Clam	0	5	0	0	0	0	0	0	0	2	0	0	0
Shrimp	1	5	1	0	0	0	0	0	0	0	5	0	0
Crab	2	0	0	1	0	0	0	0	0	3	1	0	0
Bird	0	0	1	2	0	0	0	0	0	0	0	0	0
Other	0	1	0	2	0	0	0	0	0	0	2	0	1
Water													
Unspecified	38	80	9	3	0	0	0	1	0	1	1	3	0
Ocean or estuary	0	4	3	0	0	0	0	0	0	3	1	9	0
Lake or stream	0	3	2	0	2	0	0	0	1	0	0	0	0
Sewage	28	20	0	3	0	0	1	0	0	0	0	1	0
Food	0	18	1	3	0	0	0	0	0	1	4	0	0
Culture collections	0	0	0	0	0	0	0	0	0	0	2	0	0
Other or unknown	43	18	13	2	0	1	2	9	3	3	7	3	0
Total	246	400	92	23	14	34	22	30	23	74	162	124	2

Studied at the *Vibrio* Reference Laboratory, Enteric Bacteriology Section, CDC.

Table 4. Growth of *Vibrio* cultures on TCBS agar.

Organism	Colony appearance <sup>a</sup> on TCBS (%)		Growth-plating efficiency
	Green	Yellow	
<i>V. cholerae</i>	0 <sup>a</sup>	100 <sup>a</sup>	Good
<i>V. mimicus</i>	100	0	Good
<i>V. metschnikovii</i>	0	100	May be reduced
<i>V. hollisae</i>	100	0	Very poor
<i>V. damsela</i>	95	5	Reduced at 36°C
<i>V. fluvialis</i>	0	100	Good
<i>V. furnissii</i>	0	100	Good
<i>V. alginolyticus</i>	0	100	Good
<i>V. parahaemolyticus</i>	99	1	Good
<i>V. vulnificus</i>	90 <sup>b</sup>	10 <sup>b</sup>	Good
<i>V. carchariae</i>	0	100	Good
<i>V. cincinnatiensis</i>	0	100	Very poor
"Marine vibrios"	Variable	Variable	Variable

<sup>a</sup>Percentage of strains that produce green colonies and yellow colonies, respectively.

<sup>b</sup>The original report describing this species gave the percentage positive for sucrose fermentation as 3%. At the CDC *Vibrio* Laboratory, about 15% of the strains have been sucrose positive.

which cause extraintestinal infections (Table 2); however, this division is not absolute. The pandemic strain of *V. cholerae* (serogroup O1, cholera-toxin<sup>+</sup>) seems to be well-adapted to the human intestinal tract, and it is seldom found at other sites (Table 3). This is in contrast to other serotypes of this species (*V. cholerae* non-O1) which occur at a variety of other sites. Similarly, *V. parahaemolyticus* also occurs mainly in gastroenteritis. Most of the reports of wound infections and septicemia due to this organism were really due to *V. vulnificus*, which had not been described at that time. *V. vulnificus* is an important cause of (often fatal) septicemia and wound infections (Blake et al., 1979, 1980b; Hollis et al., 1976). *V. damsela* (Love et al., 1981) also appears to cause human wound infections (Morris et al., 1982). *V. alginolyticus* (Blake et al., 1979) has been isolated from several types of soft tissue infections. *V. metschnikovii* is usually an environmental organism (Lee et al., 1978a), but has been isolated from a case of peritonitis in a patient with an inflamed gallbladder (Jean-Jacques et al., 1981), and from a few other specimens. Most of the infections caused by the marine species of *Vibrio* are associated with exposure to seawater.

**THE MARINE ENVIRONMENT** Since detailed taxonomic studies allowing precise identification of the species of *Vibrio* and *Photobacterium* isolated from this ecological niche are just beginning, generalizations concerning habitats and distribution of most species cannot be made. Baumann and Baumann (1981) (see Table 2 in their review) summarized the information available that appeared to be based on sound taxonomic criteria. They found that strains from

geographically diverse locations, which have been assigned to the same species on the basis of phenotypic similarities, were found to have in vitro DNA-DNA homologies greater than 80%. Most of the remaining species of marine enterobacteria and the nonfermentative marine eubacteria were isolated from the open ocean 10–35 miles off the coast of Oahu, Hawaii, at depths ranging from surface waters to 1,300 (Baumann and Baumann, 1981). Most of the sites had little or no obvious terrestrial contamination. Several of these *Vibrio* species have not been reported from other locations.

There are good data on the habitats of the bioluminescent bacteria, some of which are able to enter into symbiotic association with marine animals. A number of studies have appeared dealing with the ecology of luminous bacteria in seawater off the coast of San Diego, California (Ruby and Nealson, 1978), the Eastern Mediterranean, and the Gulf of Elat (Shilo and Yetinson, 1979; Yetinson and Shilo, 1979), as well as from two locations in the open ocean in the North Atlantic and over the Puerto Rico Trench (Ruby et al., 1980). The results of these studies show a species-specific pattern influenced by season, depth, geographical locale, and salinity. These investigations are important because they represent ecological studies in which marine bacteria could be identified to the species level. Shilo and Yetinson (1979) were able to observe a correlation between the ecology of the luminous organisms and their physiological attributes.

**SIMPLE METHODS TO DETERMINE THE NaCl CONTENT OF ENVIRONMENTAL WATER** Since many of the species of *Vibrio* and *Photobacterium* are

found in salt and estuary water, it is often desirable to quickly estimate salinity in order to decide where to sample, especially for inland waters. Two simple methods are given below that are particularly useful in the field. A quantitative method can be used when the specimen is returned to the laboratory.

#### Method 1—Silver Nitrate ( $\text{AgNO}_3$ ) Method

This method takes advantage of the fact that  $\text{NaCl}$  is usually the main salt that contributes  $\text{Na}^+$  in environmental water samples.  $\text{Ag}^+$  reacts with  $\text{Cl}^-$  to form a dense white precipitate.

Dispense 1.0 ml of 1% silver nitrate solution (see below) into a disposable  $13 \times 100$  mm test tube. Take a 0.1-ml sample of the liquid whose  $\text{Na}^+$  content is desired and add it to the tube. A white precipitate will form almost immediately. The amount of precipitate will be proportional to the amount of  $\text{NaCl}$  present in the sample. An alternative method is to add a drop of the silver nitrate solution to the water and observe the intensity of the white precipitate formed.

This method actually measures the  $\text{Cl}^-$  content of the liquid, but for bacteriological media and water samples taken near the coast, this is close to the concentration of  $\text{NaCl}$ .

Silver nitrate solution,	1%
Silver nitrate ( $\text{AgNO}_3$ )	10 g
Water	1 liter

Dissolve the  $\text{AgNO}_3$  in the water. Store in a brown bottle (or in a clear glass bottle that must be kept in the dark). In the presence of light, a brown-black precipitate will form. It is sometimes convenient to dispense 1-ml volumes into a rack of screw-cap tubes and store them in the dark until sampling is done.

#### Method 2—Dissolved Solids Meter

There are number of inexpensive pocket meters that measure conductivity or total dissolved solids and provide a convenient way to quickly determine the approximate  $\text{Na}^+$  content of media and water samples.

Insert the pocket meter into the liquid sample and obtain a reading. Check this against a calibration curve based on different  $\text{NaCl}$  concentrations, and read the concentration of dissolved ions, which should approximate the concentration of  $\text{NaCl}$ .

## Isolation

Much has been written about the isolation of both *Vibrio* and *Photobacterium* species. Two different sets of methods have been used, depending on whether the specimen (sample) was from a human clinical specimen or from the marine environment. Although species of *Vibrio* and *Photobacterium* will often grow on both kinds of media, the low  $\text{Na}^+$  content (0.5 to 0.85%) of some media will prevent growth of some marine species. Only limited data exist on the growth of the species isolated from human diseases on many of the media traditionally used by marine microbiologists. In spite of these differences, several media are useful for all types of research with these genera.

## Artificial Seawater Recipes

It is often desirable to have a medium with all of the inorganic constituents of seawater, but without organic compounds and toxic contaminants. Several artificial seawaters are useful for this purpose.

#### ARTIFICIAL SEAWATER #1—MACLEOD'S FORMULA

This artificial seawater is very easy to prepare. Its formula does not contain several of the ingredients found in other formulas for artificial seawater (some of these are trace metals that are probably present in the distilled water added). One advantage of this formula is that it does not form a precipitate when it is autoclaved. This medium should be satisfactory for most work with *Vibrio* and *Photobacterium*.

#### Artificial Seawater #1—MacLeod's Formula (CDC Medium 1425)

$\text{NaCl}$	23.38 g (0.4 Molar)
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	24.65 g (0.1 Molar)
$\text{KCl}$	1.49 g (0.02 Molar)
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2.94 g (0.02 Molar)
Water	1 liter

Dissolve the ingredients in the water; a crystal-clear solution with a pH of about 6.6 will result. Dispense into screw-cap bottles or tubes. Autoclave at  $121^\circ\text{C}$  for 15 min. The final solution should be colorless and crystal clear. Store at room temperature.

#### ARTIFICIAL SEAWATER #2—"INSTANT OCEAN"

This is an artificial seawater that is prepared from a commercial packaged mix. It contains all of the ingredients found in seawater. Instant Ocean is available from Aquarium Systems, 33208 Lakeland Blvd., Eastlake, Ohio 44094, but it can also be found in many pet stores, particularly those that stock supplies for marine aquariums. It comes in several sizes, including a 1.6-lb package.

#### Artificial Seawater #2—Instant Ocean

Instant Ocean	40 g
Distilled water	1 liter
pH	about 8.6.

We find that this mix contains a variable amount of water that has been absorbed from the air. Forty grams of Instant Ocean per liter should give approximately normal oceanic salinity; more precise work may require a different amount that can be calculated based on "dry weight" or specific gravity.

Add the Instant Ocean to the water. Stir on a magnetic mixer for 15 to 20 min until all the salt dissolves. A colorless solution will result (sometimes with a slight amount of turbidity). If a sterile solution is needed, dispense and autoclave at  $121^\circ\text{C}$  for 15 minutes. Store at room temperature.

There are also several good general plating media for isolation of *Vibrio* and *Photobacterium*

(see below). Marine agar is a nonselective medium, and essentially all vibrios will grow on it. TCBS agar is a very selective medium, and many vibrios strains will grow on it. Most other bacteria are inhibited.

### Marine Agar

Marine agar is a useful medium for the isolation and growth of organisms whose natural habitat is marine or other environments with a high content of  $\text{Na}^+$ . It is particularly useful for the species of *Vibrio* and *Photobacterium* that require  $\text{Na}^+$  in amounts higher than the usual content of bacteriological media, which is 0.5 to 1.0%.

#### Marine Agar

The formula used by Difco Laboratories (Catalog no. 0979) is given below; it is available as a dehydrated powder.

Peptone (Bacto)	5 g
Yeast extract	1 g
Ferric citrate	0.1 g
Sodium chloride	19.45 g
Magnesium chloride	8.8 g
Sodium sulfate	3.24 g
Calcium chloride	1.8 g
Potassium chloride	0.55 g
Sodium bicarbonate	0.16 g
Potassium bromide	80 mg
Strontium chloride	34 mg
Boric acid	22 mg
Sodium silicate	4 mg
Sodium fluoride	2.4 mg
Ammonium nitrate	1.6 mg
Disodium phosphate	8 mg
Water	1 liter
Agar	15 g

Add 55.1 g of Difco marine agar (or the recipe given above) to the distilled water and heat to boiling. pH  $7.6 \pm 0.2$  at  $25^\circ\text{C}$ . Dispense into tubes or bottles and autoclave at  $121^\circ\text{C}$  for 15 minutes. A brown gelatinous precipitate is produced during autoclaving which settles to the bottom. Cool the flask to  $45\text{--}50^\circ\text{C}$  and mix the flask contents to disperse the brown precipitate as much as possible. Pour into petri dishes and allow to harden and cool to room temperature. The bottom of the plates will contain some brown precipitate, which should not be confused with bacterial growth.

### TCBS (Thiosulfate-Citrate-Bile Salts-Sucrose) Agar

This medium is extremely useful for isolating *Vibrio cholerae* and *V. parahaemolyticus* from diarrheal stool specimens. It is also used as a general isolation-plating medium for vibrios from clinical specimens and from the environment (Table 4, Fig. 5). Some *Vibrio* species do not grow or grow poorly on TCBS agar. Dehydrated TCBS medium is available from BBL, Eiken, Oxoid, and Gibco. These manufacturers may use different peptones (polypeptone by

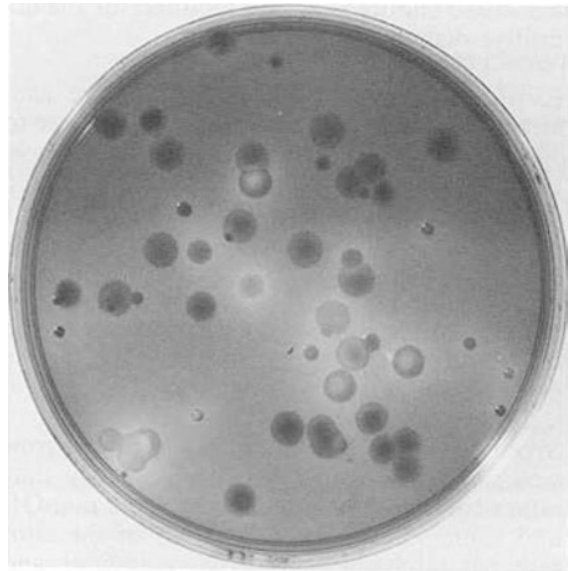


Fig. 5. Growth of vibrios on TCBS agar; 0.01 ml of seawater collected about 10 m from shore was plated and incubated for 3 days at ambient temperature (about  $25^\circ\text{C}$ ); note the different sizes, shapes, and colors of the colonies.

BBL, Proteose Peptone no. 3 by Difco, Eiken Peptone by Eiken, etc.), and the selectivity of the medium will vary from manufacturer to manufacturer and from lot to lot from the same manufacturer. Quality control should be done on each new lot number.

#### TCBS Agar

This is the recipe given by BBL for their TCBS agar.

Yeast extract	5 g
Pancreatic digest of casein	5 g
Pancreatic digest of animal tissue	5 g
Sodium citrate	10 g
Sodium thiosulfate	10 g
Oxgall, dehydrated	5 g
Sodium cholate	3 g
Sucrose	20 g
Sodium chloride	10 g
Ferric citrate	1 g
Thymol blue	40 mg
Brom thymol blue	40 mg
Agar	14 g
Water	1 liter

Suspend 88 grams of the dehydrated powder in 1 liter of cold water and heat gently with frequent agitation to boiling to dissolve the agar. pH  $8.6 \pm 0.2$ . Do not overheat or autoclave. Cool to  $45\text{--}50^\circ\text{C}$  and pour into petri dishes.

There are also several good liquid media for isolating or growing vibrios. One recipe is given below.

### Alkaline Peptone Water (CDC Medium 1494)

This medium is used for enriching for *Vibrio cholerae* and other *Vibrio* species. Species of



*Vibrio* grow better than most other organisms at this high pH. They also tend to grow better than other organisms at the aerobic surface of the liquid. Enrichment in alkaline peptone water is usually followed by plating a loopful from the surface onto TCBS agar or onto similar plating medium selective for *Vibrio*. The content of NaCl in alkaline peptone water is not standardized, but is usually 0.5 to 1%. It can be increased to 2% to allow better growth of the marine vibrios. If no NaCl is added, it becomes much more selective for *V. cholerae* and *V. mimicus* (see alkaline peptone water—saltless). The type of peptone used in the medium has also varied depending on several factors including local availability. The formula given below is the one used in our laboratory for many years. Other formulations are probably equally effective, but we have no experience with them.

#### CDC Medium 1494

Peptone (Bacto)	10 g
Sodium chloride	5 g
Water	994 ml
Sodium hydroxide, 1 N (see below)	

Dissolve the peptone and sodium chloride in the water. Insert a pH electrode and add 1N NaOH dropwise until the pH has risen to 8.4; about 6 ml will be required. Dispense and autoclave at 121°C for 15 min. The final medium will be clear and amber colored.

## Isolation—Clinical Specimens

### Laboratory Routine for *Vibrio* Work

Many of the techniques used in clinical microbiology and enteric bacteriology laboratories work well with the genus *Vibrio*. However, there are some specialized items that are recommended for laboratories that often isolate and identify *Vibrio* species (Barua and Burrows, 1974; Benenson et al., 1964; Hugh and Sakazaki, 1972; Morris et al., 1979; Wachsmuth, 1984; World Health Organization, 1983) and for clinical laboratories that want to increase their capability. Two factors can complicate the isolation and identification of *Vibrio*. Sometimes *Vibrio* cultures will not grow well on the highly selective media used to isolate “enteric pathogens.” The other factor is that the halophilic species of *Vibrio* need added NaCl for optimum growth and activity (Baumann and Schubert, 1984), and several common laboratory media have suboptimum amounts of Na<sup>+</sup> (less than 0.5% NaCl). Solutions to these problems are discussed below.

### Collection, Transport, and Storage of Specimens

**EXTRAIESTINAL SPECIMENS** The usual procedure for collecting and processing these speci-

men (blood, wound, tissue, etc.) is followed. There are no special procedures for *Vibrio*.

**STOOL SPECIMENS** Stool specimens should be collected early, preferably within the first 24 hours of illness, and before the patient has received any antimicrobial agents.

**TRANSPORT** Whenever possible, stool or rectal swab specimens should be inoculated on isolation plates with minimal delay. Viability of *Vibrio* species is well maintained at the alkaline pH of typical feces from cholera patients’ rice-water stools, but is unpredictable in formed stools. *Vibrios* are very susceptible to desiccation, so specimens must not be allowed to dry.

When there will be a delay in plating a culture (especially when it must be transported by courier), rectal swabs or fecal material should be placed in alkaline peptone water or into Cary and Blair semisolid transport medium, which maintains viability of *Vibrio* cultures for up to 4 weeks. Buffered glycerol-saline, often used in enteric bacteriology, is an unsatisfactory transport medium even for short periods. Tellurite-taurocholate-peptone broth has been extensively used with success as an “enrichment transport” medium at the International Center for Diarrheal Diseases Research in Dhaka, Bangladesh, where specimens collected in the field are generally plated within 12 to 24 hours. In the absence of available suitable transport media, strips of blotting paper may be soaked in liquid stool and inserted into airtight plastic bags. Specimens collected in this way may remain viable for up to 5 weeks.

**STORAGE AND SHIPMENT** Specimens in transport media may be shipped to the laboratory without refrigeration, but all normal precautions and shipping regulation must be followed.

### Overall Plan for Isolation

Table 5 gives four different approaches to the isolation of *Vibrio*. The approach adopted by a particular laboratory will probably depend on the frequency with which *Vibrio* cultures are encountered. The routine use of TCBS medium brings immediate attention to a possible *Vibrio* isolate, but its routine use is not cost effective. In an 18-month study, M. T. Kelly (see Farmer et al., 1985) found that every *Vibrio* isolated from TCBS medium was also detected on other plating media which were screened for oxidase-positive colonies. Other negative factors include the fact that different lots of commercial TCBS medium vary in their selectivity, and some species or strains of *Vibrio* do not grow well on TCBS.

Figure 6 gives an overall plan for using special methods to enhance the *Vibrio* isolation rate. One simple procedure will probably result in a higher isolation rate for *Vibrio*. Hemolytic colonies on sheep blood agar plates can be tested for their oxidase reaction, which will detect strongly hemolytic colonies of *Aeromonas* and some *Vibrio* species. This will also detect some weakly hemolytic *Vibrio* and *Plesiomonas* cultures. Oxidase testing can also be done on non-hemolytic colonies (see below). A latex agglutination test (Fig. 6) can be useful as a rapid screening method for detecting *V. cholerae* but a positive culture is always required for the definitive diagnosis of cholera.

**EXTRAIESTINAL SPECIMENS** These are usually processed with no particular attention to *Vibrio*. The *Vibrio* species of medical importance grow well on blood agar and many also grow on MacConkey agar. On blood agar strains of *V. cholerae* have a characteristic morphology (Fig. 7) and are usually strongly hemolytic (except for the classical biogroup). However, a more thorough search for *Vibrio* isolates can be done with

oxidase testing or by including a plate of TCBS medium.

**STOOL SPECIMENS** Cultures of *Vibrio* will grow well on blood agar (Fig. 8) where they may either be: beta-hemolytic (*V. cholerae* non-O1, and some *V. cholerae* O1 strains of the eltor biotype), alpha-hemolytic *V. vulnificus* and many others), or non-hemolytic. They usually grow on MacConkey agar (sometimes with a reduced plating efficiency) and will appear as colorless (lactose-

Table 5. Four approaches for the isolation of *Vibrio* cultures from clinical specimens.

1. Use normal procedures and make no special effort to search for *Vibrio*.
2. Use normal procedures and look for oxidase-positive colonies on plating media, especially on blood agar.
3. Incorporate TCBS agar as an extra plate for stool cultures, and also for other likely specimens such as wounds, blood, eye, and ear.
4. Use other special media and procedures to enhance the isolation of *V. cholerae*, *V. parahaemolyticus*, and other *Vibrio* species.

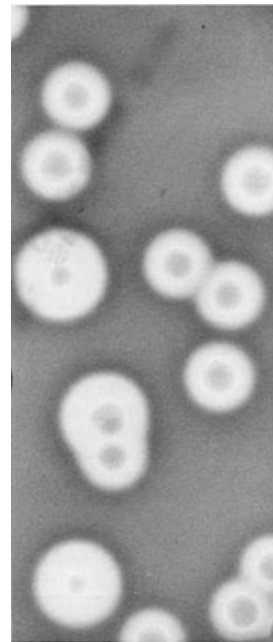


Fig. 7. Strong hemolytic reaction of the Gulf Coast strain of *Vibrio cholerae* on sheep blood agar plates (overnight incubation at 36°C); note that the small colony type has a much larger zone of hemolysis than the large colony type.

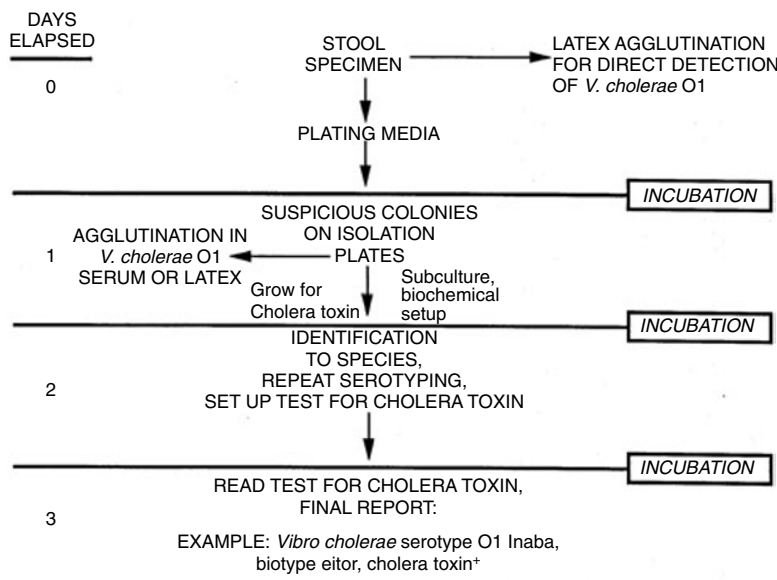
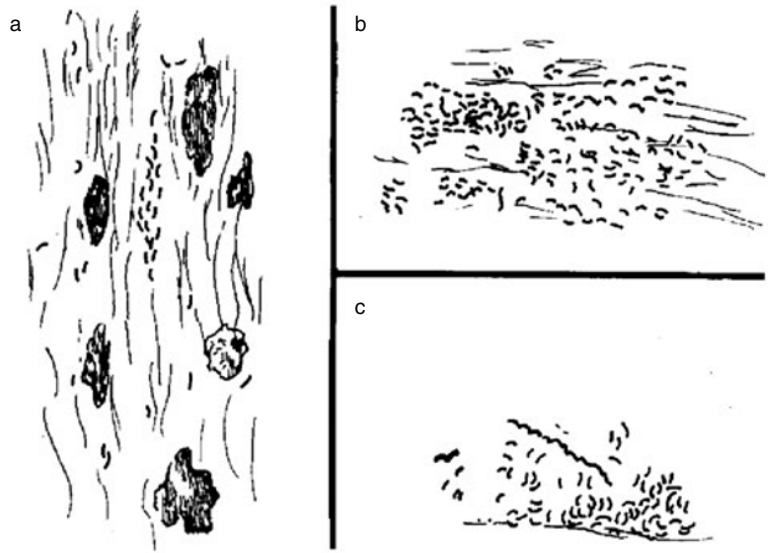


Fig. 6. Overall plan and special methods for the isolation and identification of *Vibrio cholerae* and other *Vibrio* cultures.

Fig. 8. Variation in the size and shape of *Vibrio cholerae* cells taken directly from cholera patients and laboratory cultures as reported by Robert Koch in 1883; note the curved rods that led Koch to use the name “comma bacillus.” (a) Intestinal content of a cholera patient. (b) Rice-water stool of a cholera patient after 2 days incubation on moist clothing. (c) Growth from meat-broth laboratory culture (note long spiral forms). (All redrawn from Koch’s original figures as reproduced on p. 100 of Pollitzer, 1959.)



negative) colonies. Oxidase testing can be done on colonies grown on blood agar and on lactose-negative colonies on selective media; however, lactose-positive colonies from selective media can give false-negative oxidase reaction. *Vibrio* cultures often do not grow well on the more selective enteric plating media.

### Oxidase Testing of Colonies from Primary Plates

This appears to be a cost-efficient method for the detection of *Vibrio* cultures without having to add an additional plating medium such as TCBS agar.

#### Oxidase Tests

**Method 1.** An isolated colony on a blood agar plate is touched and spot tested for oxidase reaction by Kovacs’ method. A total of 50 to 10 colonies are tested.

**Method 2.** Growth from a crowded area is taken up with a cotton swab and smeared onto the filter paper soaked with Kovacs’ oxidase reagent.

**Method 3.** A drop of Kovacs’ oxidase reagent is added to an area of the blood agar plate where the colonies are crowded but still separated. The drop should cover 50 to 100 colonies. Oxidase-positive colonies turn purple within 1 minute.

If oxidase-positive colonies are present in methods 2 or 3, individual isolated colonies (not previously touched or exposed to the reagent) from an area of less crowding are tested for their oxidase reactions as described in method 1. Since most specimens will have no oxidase-positive colonies (other than *Pseudomonas aeruginosa*), this method reduces the number of colonies which require individual screening. Oxidase-positive colonies (other than *P. aeruginosa*) detected by the above method are then identified to species. This method has another advantage because it also detects cultures of *Aeromonas* and *Plesimonas*. Oxidase testing should improve the isolation rate, but it may not be very cost effective in some geographical locations because of low yields of *Vibrio*, *Plesimonas*, and *Aeromonas*.

### Specialized Media and Methods for *Vibrio* Isolation

**USE OF TCBS AGAR** The advantage of using TCBS agar is that it increases the laboratory worker’s awareness of suspect *Vibrio* colonies, and usually results in an increased number of isolates. Many laboratories near oceans or salty areas use TCBS agar as a plating medium for stool and other specimens (Bonner et al., 1983). It is recommended as the single best medium to detect *Vibrio* from human clinical specimens. [Table 4](#) gives some information about the growth of the *Vibrio* species on TCBS agar. This medium is particularly useful for isolating *V. cholerae* or *V. parahaemolyticus* from feces. There is considerable variability in the selectivity of TCBS from different manufacturers and quality control of each new lot is essential.

**SPECIAL EFFORTS TO ISOLATE *VIBRIO CHOLERA*E AND OTHER *VIBRIO* SPECIES FROM FECES** [Fig. 6](#) is a schema adapted from Wachsmuth et al. (Wachsmuth et al., 1980) and Furniss et al. (Furniss et al., 1978) for the isolation of *V. cholerae* from feces. It should also be useful in isolating other *Vibrio* cultures from feces and other specimens. TCBS agar and enrichment in alkaline peptone water select for *Vibrio* species. The main disadvantage of this approach is that these methods are in addition to the usual laboratory routine, and may cause much additional work with a poor yield of specimens positive for *Vibrio*.

**MICROSCOPIC EXAMINATION OF GROWTH** In 1883, Koch noted the appearance of small curved rods in the rice-water stools of cholera patients ([Fig. 8](#)). These typical curved rods were not seen in feces of patients without cholera. A number of

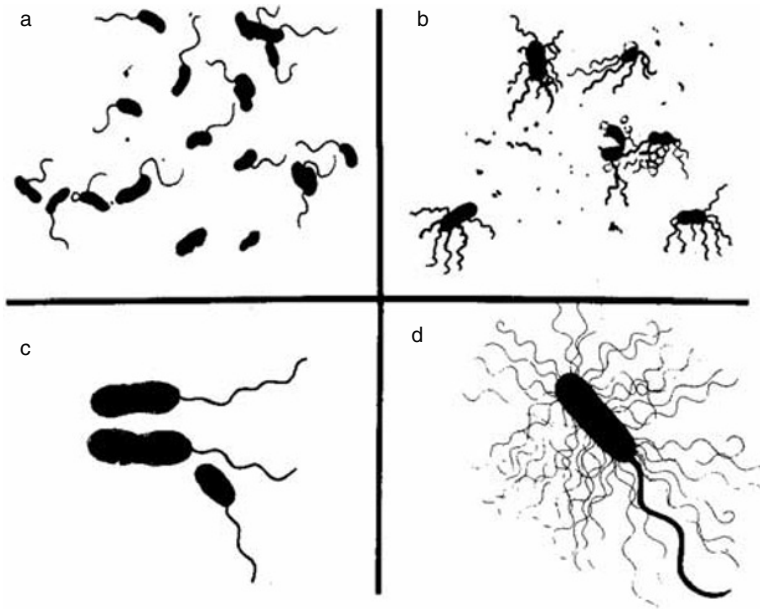


Fig. 9. Cellular morphology of *Vibrio cholerae*, *V. parahaemolyticus*, and *V. alginolyticus*. (a) *V. cholerae* from an 18-h culture on nutrient agar (Van Ermengen cilia stain). (b) Flagella stain of *V. parahaemolyticus*. (c) Electron micrograph of *V. parahaemolyticus*; note single-sheathed polar flagellum. (d) Electron micrograph of *V. alginolyticus* grown on solid media; peritrichous flagella are present; note their different size and shape as compared to the sheathed polar flagellum. (From Farmer et al., 1985.)

other authors have made the same observation and commented on the possible usefulness of this observation. *Vibrio cholerae* has considerable variability in its cellular morphology (Fig. 8), which includes typical curved rods, straight rods, short noncurved rods, and “involution forms” (Fig. 8C). Often these can all be seen in the same culture. For this reason, microscopic examination of feces or of cultures does not have a prominent role today. However, the finding of typical curved forms can be used as presumptive evidence for the presence of *Vibrio*. *Vibrio* cultures grown in liquid media have polar flagella, but many strains have peritrichous flagella when grown on solid media (Fig. 9). However, flagella stains are impractical for routine identification.

## Isolation—Marine and Environmental Samples

Enrichment Cultures (from Baumann and Baumann, 1981)

Most enrichment cultures are incubated at room temperature (18–22°C), while cultures on petri plates are incubated at ambient temperature or 25°C. These temperatures are primarily a matter of convenience and can be modified (usually reduced, as in the case of the luminous bacteria) to suit the particular needs of the investigator.

Carbon sources are added to enrichments at a concentration of 0.1–0.2% (w/v for solids and v/v for liquids). For the cultivation of amino acid-requiring organisms, basal medium (medium “BM” of Baumann and Baumann, 1981) is supplemented with 1 mg/liter each of L-alanine,

L-arginine, L-asparagine, L-aspartate, L-cysteine, L-glutamate, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. The amino acids are filter-sterilized and added to the autoclaved medium.

The carbon—energy source (unless labile or volatile) is added to the double-strength medium BM prior to autoclaving. Filter-sterilized labile or volatile compounds are added to the basal medium with added agar (medium BMA of Baumann and Baumann, 1981), which has been cooled to about 41°C prior to the pouring of plates. Some volatile substrates (geraniol, *n*-hexadecane, naphthalene, and phenol) are not added to the medium but are placed on sterile filter paper in the lids of the inverted petri dishes, then incubated in air-tight containers. It should be stressed that the medium of MacLeod based in artificial seawater (1968) may not be suitable for growing some marine organisms that may require additional mineral components. A number of different artificial seawater formulations have been compiled by Kinne (1976). Although many marine bacteria are not adversely affected by Tris buffer, the compound may prove toxic for some strains.

BM Medium (Basal Medium of Baumann and Baumann, 1981)

Tris HCl (pH 7.5) (see below)	15.8 g (100 mM)*
Ammonium chloride (NH <sub>4</sub> Cl)	1 g (19 mM)
Potassium phosphate (K <sub>2</sub> HPO <sub>4</sub> · 3H <sub>2</sub> O)	57 mg (0.33 mM)
Ferrous sulfate (FeSO <sub>4</sub> · 7H <sub>2</sub> O)	28 mg (0.1 mM)
Water	500 ml

Artificial seawater (MacLeod formula) 500 ml  
(see "Isolation," this chapter)

Or use 7.9 grams for 50 mM (Baumann and Baumann, 1981)

#### BMA Medium (Basal Medium Agar of Baumann and Baumann, 1981)

Prepare 500 ml double-strength BM medium, add the carbon source (if it can withstand autoclaving), autoclave, and cool. If the carbon source is heat labile, filter-sterilize and add aseptically to cooled BM medium. Prepare 500 ml of a double-strength agar solution (40 g refined agar per liter), autoclave, and cool. Mix the BM plus carbon source with the agar solution and pour onto plates.

#### Isolation from Seawater (Baumann and Baumann, 1981)

It is essential that seawater be collected aseptically using sterile samplers. In general, this presents little difficulty when samples of surface waters are collected. A convenient way of obtaining seawater from different depths is by use of the Niskin butterfly sampler (General Oceanics, Miami, FL, USA). The collected samples can be used either for enrichment cultures or for direct isolation.

**DIRECT ISOLATION** Samples of seawater (5–300 ml) are filtered through 0.22- or 0.45- $\mu\text{m}$  nitrocellulose filters which are placed on petri plates containing either a complex medium (marine agar or a similar nonselective medium) or BMA medium with 0.1% of the carbon and energy source. The size of the filtered sample will depend on the source of the seawater and the composition of the medium used for direct isolation. After an incubation of 2–10 days at 25°C, colonies are picked and restreaked on homologous media. *V. nigripulchritudo* can be frequently obtained by direct isolation on plates containing BMA with 0.2% lactose, which will also often yield *Alteromonas macleodii*.

Many agar decomposers produced a broad but barely perceptible indentation in the agar surrounding the colony. Consequently, it is advisable to streak a pure culture on BMA medium without an added carbon and energy source and containing appropriate supplements for growth-factor-requiring organisms. Growth on this medium indicates that the isolate is able to utilize agar. Organisms in the marine environment apparently do not utilize Tris buffer as a carbon and energy source, although some organisms appear to utilize this compound as a poor nitrogen source.

**ENRICHMENT CULTURES** For aerobic enrichments, 500 ml of seawater is added to a sterile 2-liter Erlenmeyer flask containing 25 ml of 1 M

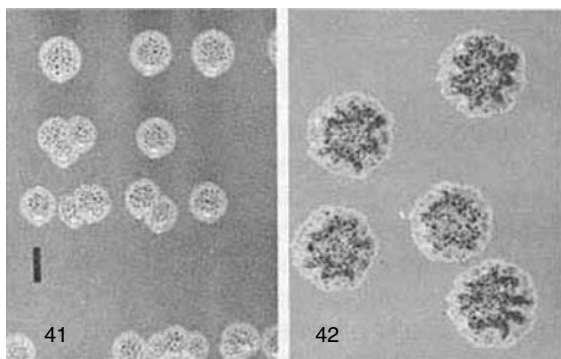


Fig. 10. Colonial morphology of *Vibrio nigripulchritudo*; note the granules of blue-black pigment embedded in the colonies. (From Baumann and Baumann, 1981.)

Tris-HCl (pH 7.5), 0.5 g  $\text{NH}_4\text{Cl}$ , 38 mg  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 14 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.5–1.0 g of the carbon and energy source (acidic or basic carbon sources may require readjustment of the pH to 7.5). Depending on the source of the sample and the organic compound used to support growth, both the sample volume and the volume of the concentrated solution may be increased or decreased. The flasks are observed for signs of growth for up to 10 days, and then they are streaked on BMA medium containing 0.1% of the same carbon and energy source as was used in the enrichment. Enrichments containing 0.2% chitin, incubated aerobically for 5–10 days, often contain a blue-black sediment associated with the chitin particles. Such enrichments, when streaked on medium BMA containing 0.2% lactose, usually yield *V. nigripulchritudo*, a chitin-decomposer which forms colonies containing crystals of blue-black pigment (Fig. 10) (Baumann et al., 1971b). *V. alginolyticus* can be readily obtained from aerobic enrichments containing 0.5% yeast extract and 0.5% tryptone (instead of the single organic carbon and energy source). When streaked on marine agar or a similar medium, this organism swarms in a manner similar to that of *Proteus*. Single colonies can be obtained by streaking on either a complex medium containing 4% agar or on minimal medium such as BMA with 0.2% glycerol.

**ISOLATION FROM SURFACES AND INTESTINAL CONTENTS OF FISH** Sterile cotton-tipped applicator sticks are used to swab the gills, mouth, and rectal region as well as other surfaces of the fish. An agar plate is then streaked to give isolated colonies. A 2–3 cm portion of the fish intestine is dissected and placed onto a sterile petri plate. Gentle pressure with sterile forceps or an applicator stick generally forces out some of the intestinal contents, which are streaked on plating medium. In both cases the plates are

incubated for 1–4 days and observed for colonies, which are then purified by streaking on homologous medium. Some marine strains swarm extensively on complex media making it impossible to isolate single colonies. Swarming can be prevented by raising the concentration of agar to 4%.

### Isolation of Bioluminescent Bacteria (Baumann and Baumann, 1981)

These organisms are common in the marine environment. The detection of luminescence by a simple visual examination poses a number of problems since the intensity of the emitted light varies greatly with different isolates and is also affected by the cultivation medium as well as by the age of the cells. A satisfactory medium used for the observation of luminescence is LM medium (Baumann and Baumann, 1981). Strains are streaked on this medium and incubated at 15°C and 25°C with periodic examinations at 12–36 h after streaking. In many strains, luminescence is relatively dim and short-lived so that frequent observation (every 3 h) is suggested. It is important to have plates with isolated colonies since, in the case of many dim strains, only the isolated colonies luminesce. Examination for luminous colonies on plates containing LM medium should be done in complete darkness, and the eyes should be dark-adapted for at least 10 min. The picking of luminous colonies is greatly facilitated by the use of sterile toothpicks and a low-intensity light bulb (5–10 watts) connected to a rheostat (Cosenza and Buck, 1966). First, in total darkness, an area of luminescence is observed, and a sterile toothpick is positioned roughly over the site. The current is then switched on and the intensity of the light is gradually increased until the specimen is just barely visible. By fixing one's eyes on the luminous spot and gradually increasing the intensity of the light it becomes apparent which area contains the luminous organisms. The toothpick is quickly touched to this site; a plate of LM medium is inoculated; and the light is quickly turned off. By using this procedure, the investigator's eyes do not have to be repeatedly dark-adapted and, more importantly, it is possible to actually see the area from which the inoculum is picked.

LM Medium (Luminous Medium of Baumann and Baumann, 1981)

Tris HCl (pH 7.5)	7.9 g (50 mM)
Yeast extract	5 g
Tryptone	5 g
Calcium carbonate (CaCO <sub>3</sub> )	1 g
Agar	20 g
Glycerol	3 g
BM medium	1 liter

**ISOLATION FROM SEAWATER** In some coastal waters, the concentration of luminous bacteria may be sufficient to allow detection in a 0.1 to 1-ml sample spread onto a plate of LM medium, TCBS medium, or marine agar. With larger volumes of seawater, aliquots of up to 300 ml may be filtered through 0.22 or 0.45- $\mu$ m nitrocellulose filters which are subsequently placed onto petri plates containing one or more of these media. Since crowded conditions tend to inhibit luminescence and since nonluminous bacteria greatly outnumber the luminous isolates, it is important that the filter contain a relatively sparse bacterial population. It is not recommended that the soft agar overlay method be used for the enumeration of luminous bacteria since brief exposure to 41°C (the temperature of the molten agar used for the overlay) may kill some strains of *Photobacterium phosphoreum* and *V. logei*.

**ISOLATION FROM SURFACES AND INTESTINAL CONTENTS** Fresh squid and octopus, which have been kept on ice in fish markets, often have luminous spots when examined in the dark; however, luminous spots are rare on fresh fish. The isolation of luminous organisms from such specimens, from the surfaces of marine animals, as well as from the intestinal contents of fish, is done as described above. In general, the intestinal contents of fish have either a relatively large population of luminous bacteria or none (Baumann and Baumann, 1981).

**ISOLATION BY ENRICHMENT** This procedure is given in Baumann and Baumann (1981) and based on some suggestions of M. Doudoroff. In some cases, visible regions of luminescence can be obtained on fresh fish, squid, or octopus by half-submerging the specimen in a shallow layer of artificial seawater and incubating for 10–18 h at 12–15°C. The luminous sites are touched with sterile toothpicks and inoculated onto plates of LM medium or other media as previously described. This method is relatively specific for *P. phosphoreum*.

### Stock Cultures and Methods for Preservation

In the Vibrio Laboratory at CDC, a “permanent frozen stock” is made by suspending growth from a blood agar or marine agar plate in skim milk (10%) which is transferred to a plastic vial and frozen and stored at –70°C. A “working stock” is also prepared. *V. cholerae*, *V. mimicus*, and other nonhalophilic species are grown in screw-cap tubes of working-stock media; halophilic vibrios are grown in marine semisolid medium (marine broth with 0.3% agar added). After the strain has grown well (usually 24 h) a

thin layer of sterile mineral oil is added to cover the top of the agar column to prevent evaporation and drying. We do not transfer these working cultures. The occasional strain that dies is replaced from the permanent frozen stock. Tubes are stored in divided boxes at room temperature in the dark. Do not store stock cultures in the refrigerator because this kills many strains. The only exception is for strains that do not grow well above 20°C; they are stored in the refrigerator. Some isolates of *Vibrio* acquire nutritional requirements after prolonged cultivation and frequent transfer on marine agar. Baumann and Baumann (1981) suggest maintaining strains on medium BMA containing 0.2% glycerol or freeze-drying them.

Most of the strains of *Vibrio* can be freeze-dried and kept at 4°C. With a few exceptions, viable cells could be recovered after 3–5 years of storage. For the preparation of lyophils, the growth from a fresh slant is suspended in about 0.5 ml of a sterile solution consisting of one-quarter-strength artificial sea water #1 (MacLeod's formula), 5 g/liter yeast extract, and 5 g/liter peptone (adjusted to pH 7.5), and transferred into a lyophil tube which is subsequently placed in a mixture of dry ice and acetone and placed under vacuum for 10–12 hrs. The lyophils are reconstituted by suspending the powder in about 0.5 ml of an optimum growth medium and inoculating a solid medium. Growth is generally observed after 1–2 days of incubation at 25°C. For *P. phosphoreum*, a lower temperature (15–18°C) should be used.

## Identification—Clinical Isolates

Because of the varying pathogenicity and clinical significance of the different species, *Vibrio* strains isolated from clinical specimens should be identified to species. There are many different approaches to identification (for a review, see Farmer et al., 1981). One common question always needs an immediate answer: “Is the *Vibrio*-like organism isolated from this particular case of cholera-like diarrhea the pandemic strain of *V. cholerae*?” (Fig. 6).

### Ruling Out *V. Cholerae* Serogroup O1

It is often important to give a definitive answer to this question as quickly as possible. Numerous sucrose-positive (yellow colonies) on TCBS agar from a patient with rice-water stools or severe watery diarrhea warns of the possible presence of *V. cholerae* serogroup O1 (Fig. 11). Suspicious colonies are subcultured early in the day by heavy inoculation on a nonselective medium such as blood agar or trypticase soy agar which

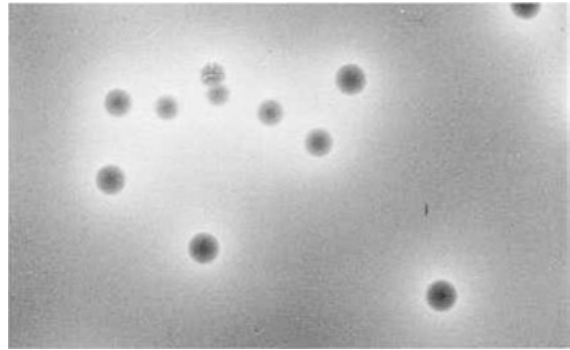


Fig. 11. Colonies of *Vibrio cholerae* on TCBS agar; yellow colonies of 2- to 3-mm diameter are present after overnight incubation at 36°C.

does not contain carbohydrates. After 5–8 h, good growth should be present which can be tested in polyvalent antisera to *V. cholerae* serogroup O1 (Fig. 6). Experienced workers use growth from the primary TCBS plate to do the agglutination. Positive agglutination is presumptive evidence of *V. cholerae* serogroup O1, and the result should be reported immediately to the physician. Biochemical testing is necessary to confirm the identification as *V. cholerae*. Local health authorities should be notified immediately, and the culture, along with information about the case, should be forwarded to the state health laboratory.

**DIRECT TESTING OF LIQUID STOOLS FOR *V. CHOLERA* O1** Jesudason et al. (1984) in India showed that there was enough O antigen in rice-water stools of cholera patients to agglutinate a reagent consisting of *Staphylococcus aureus* cells coated with antibodies to the O1 antigen. Shaffer et al. (1989) used a commercial reagent (monoclonal antibodies to serofactor “a” of the O1 antigen coated onto latex particles) for the laboratory diagnosis of cholera in the field. If sufficient O antigen is present in the liquid stool, it will cause the latex to clump (Fig. 12); this is a presumptive positive for *V. cholerae* O1. This method is particularly suited for field testing (Fig. 13) in developing countries where laboratory facilities for bacteriological analysis are usually unavailable. False negatives and false positives can occur with these two methods, so a positive culture is always needed for the definitive diagnosis of cholera.

### Biochemical Identification

**EFFECT OF MEDIA, REAGENTS, AND  $\text{Na}^+$  CONCENTRATION** Most laboratories identify *Vibrio* strains with media and tests designed to identify Enterobacteriaceae. These work well for *V. cholerae* and *V. mimicus* because they have only a low requirement for  $\text{Na}^+$ , which is fulfilled

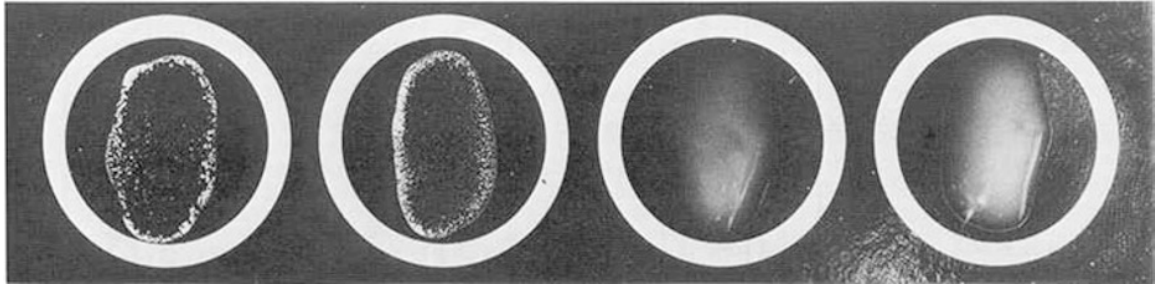


Fig. 12. Identification of *Vibrio cholerae* serotype O1 with a commercial latex reagent; two strong positive reactions are on the left, and two negative ones on the right.



Fig. 13. Laboratory diagnosis of cholera in the field using a commercial latex reagent.

by the amount of NaCl in the medium. However, most of the halophilic vibrios require much more Na<sup>+</sup> for growth and expression of various metabolic pathways. Some of the media for differential biochemical tests do not contain enough NaCl for these halophilic species. In Table 1, the standard enteric test is listed first and the percentage positive for each species is given. The percentage positive for each species is then given for a modified medium with 1% NaCl added. The table also shows that more *Vibrio* species are indole positive when the medium is changed from peptone water to heart infusion broth. More strains are Voges-Proskauer positive

when the reagent for detecting acetylmethylcarbinol contains alpha-naphthol (Berrit method). This table illustrates the pronounced effect of media and methods on the results of biochemical tests.

SCREENING AND TESTS AND COMPLETE IDENTIFICATION Table 6 gives the tests that are most helpful in dividing the 12 species of *Vibrio* that are found in clinical specimens into 6 groups; Table 7 gives the complete biochemical reactions for these 12 species. The vast majority of *Vibrio* cultures isolated from clinical specimens will be easily identified as one of the 12 species listed. Growth in the absence of added Na<sup>+</sup> (growth in nutrient broth with 1% NaCl, but no growth in nutrient broth) is the essential test for differentiating *V. cholerae* and *V. mimicus* from the other 10 species of *Vibrio* and from the marine vibrios. *V. metschnikovii* is easily differentiated because it is oxidase negative and nitrate negative. *V. cincinnatiensis* is easily differentiated because it is inositol positive. *V. hollisae* is negative for arginine dihydrolase and for lysine and ornithine decarboxylases (triple decarboxylase negative), which differentiates it from the remaining *Vibrio* species (Table 6). The remaining seven species are subdivided into the "arginine-dihydrolase-positive group," which includes *V. damsela*, *V. fluvialis*, and *V. furnissii*; and into the "lysine-decarboxylase-positive group," which includes *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. carchariae* (Table 6). The tests that are most useful for further differentiating the 12 clinical species are given in Tables 7 to 14. Several of these media and tests are specialized for vibrios and will be described in more detail later.

COMPUTER IDENTIFICATION We have developed several computer programs that have proved useful in the routine identification of *Vibrio* and *Photobacterium* isolates. The programs are based on mathematical analysis of the results of the



Table 6. Key differential tests to divide the 12 *Vibrio* species that are found in clinical specimens into six groups.<sup>a</sup>

Test	Reactions of the species in: <sup>b</sup>											
	Group 1		Group 2		Group 3		Group 4		Group 5		Group 6	
	<i>V. cholerae</i>	<i>V. mimicus</i>	<i>V. metschnikovii</i>	<i>V. cincinnatiensis</i>	<i>V. hollisae</i>	<i>V. damsela</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>V. alginolyticus</i>	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. carchariae</i>
Growth in nutrient broth:												
With no NaCl added	+	+	-	-	-	-	-	-	-	-	-	-
With 1% NaCl added	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase			-	+	+	+	+	+	+	+	+	+
Nitrate→Nitrite			-	+	+	+	+	+	+	+	+	+
myo-Inositol fermentation	-	-	V	+	-	-	-	-	-	-	-	-
Arginine dihydrolase					-	+	+	+	-	-	-	-
Lysine decarboxylase					-							
Ornithine decarboxylase					-				+	+	+	+

<sup>a</sup>The boxes indicate the key test results. All data are for reactions within 2 days at 35–37°C, unless otherwise specified.

<sup>b</sup>Symbols: +, most strains (generally about 90 to 100%) positive; V, strain-to-strain variation (generally about 25 to 75% positive); -, most strains negative (generally about 0 to 10% positive).

Table 7. Biochemical test results and other properties of the 12 *Vibrio* species that are found in clinical specimens.

Test <sup>a</sup>	Percentage positive for: <sup>b</sup>											
	<i>V. cholerae</i>	<i>V. mimicus</i>	<i>V. metschnikovii</i>	<i>V. cincinnatiensis</i>	<i>V. holtsiae</i>	<i>V. damsela</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>V. alginolyticus</i>	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. carchariae</i>
*Indole production (HIB, 1% NaCl)	99	98	20	8	97	0	13	11	85	98	97	100
Methyl red (1% NaCl)	99	99	96	93	0	100	96	100	75	80	80	100
*Voges-Proskauer (1% NaCl; Barritt)	75	9	96	0	0	95	0	0	95	0	0	50
Citrate, Simmons	97	99	75	21	0	0	93	100	1	3	75	0
H <sub>2</sub> S on TSI	0	0	0	0	0	0	0	0	0	0	0	0
Urea hydrolysis	0	1	0	0	0	0	0	0	0	15	1	0
Phenylalanine deaminase	0	0	0	0	0	0	0	0	1	1	35	NG
*Arginine, Moeller's (1% NaCl)	0	0	60	0	0	95	93	100	0	0	0	0
*Lysine, Moeller's (1% NaCl)	99	100	35	57	0	50	0	0	99	100	99	100
*Ornithine, Moeller's (1% NaCl)	99	99	0	0	0	0	0	0	50	95	55	0
Motility (36°C)	99	98	74	86	0	25	70	89	99	99	99	0
Gelatin hydrolysis (1% NaCl, 22°C)	90	65	65	0	0	6	85	86	90	95	75	0
KCN test (percentage that grow)	10	2	0	0	0	5	65	89	15	20	1	0
Malonate utilization	1	0	0	0	0	0	0	11	0	0	0	0
*D-Glucose, acid production	100	100	100	100	100	100	100	100	100	100	100	50
*D-Glucose, gas production	0	0	0	0	0	10	0	100	0	0	0	0
Acid production from:												
D-Adonitol	0	0	0	0	0	0	0	0	1	0	0	0
*L-Arabinose	0	1	0	100	97	0	93	100	1	80	0	0
*D-Arabitol	0	0	0	0	0	0	65	89	0	0	0	0
*Cellobiose	8	0	9	100	0	0	30	11	3	5	99	50
Dulcitol	0	0	0	0	0	0	0	0	0	3	0	0
Erythritol	0	0	0	0	0	0	0	0	0	0	0	0
D-Galactose	90	82	45	100	100	90	96	100	20	92	96	0
Glycerol	30	13	100	100	0	0	7	55	80	50	1	0
myo-Inositol	0	0	40	100	0	0	0	0	0	0	0	0
*Lactose	7	21	50	0	0	0	3	0	0	1	85	0
*Maltose	99	99	100	100	0	100	100	100	100	99	100	100
*D-Mannitol	99	99	96	100	0	0	97	100	100	100	45	50
D-Mannose	78	99	100	100	100	100	100	100	99	100	98	50
Melibiose	1	0	0	7	0	0	3	11	1	1	40	0
α-Methyl-D-glucoside	0	0	25	57	0	5	0	0	1	0	0	0

Raffinose	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	
L-Rhamnose	0	0	0	0	0	0	0	0	0	0	45	0	0	1	0	0	0	0	0	0	0	0	0	0	
*Salicin	1	0	9	100	0	0	0	0	0	0	0	4	1	1	0	0	0	0	0	0	95	0	0	0	
D-Sorbitol	1	0	45	0	0	0	0	3	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	
*Sucrose	100	0	100	100	0	5	100	100	0	5	100	99	1	1	15	50	0	0	0	0	100	100	50	50	
Trehalose	99	94	100	100	0	86	100	100	0	86	100	100	99	100	100	100	0	0	0	0	0	0	0	0	
D-Xylose	0	0	0	43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Mucate-acid production	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Tartrate-Jordan	75	12	35	0	65	0	35	0	0	0	22	95	93	84	50	0	0	0	0	0	0	0	0	0	
Esculin hydrolysis	0	0	60	0	0	0	8	0	0	0	0	3	1	1	40	0	0	0	0	0	0	0	0	0	
Acetate utilization	92	78	25	14	0	0	70	0	0	0	65	0	1	7	0	0	0	0	0	0	0	0	0	0	
*Nitrate→nitrite	99	100	0	100	100	100	100	0	0	100	100	100	100	100	100	100	0	0	0	0	100	100	100	100	
*Oxidase	100	100	0	100	100	0	95	100	100	100	100	100	100	100	100	100	0	0	0	0	100	100	100	100	
DNase (25°C)	93	55	50	79	0	75	100	100	0	75	100	95	92	50	100	0	0	0	0	0	50	100	100	100	
*Lipase (corn oil)	92	17	100	36	0	0	90	0	0	0	89	85	90	92	0	0	0	0	0	0	92	0	0	0	
*ONPG Test	94	90	50	86	0	0	40	0	0	0	35	0	5	75	0	0	0	0	0	0	0	0	0	0	
Yellow pigment at 25°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Tyrosine clearing	13	30	5	0	3	0	65	0	0	0	45	70	77	75	0	0	0	0	0	0	0	0	0	0	
Growth in nutrient broth:																									
*0% NaCl	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
*1% NaCl	100	100	100	100	99	100	100	99	100	99	99	99	100	100	100	100	0	0	0	0	0	0	0	0	
6% NaCl	53	49	78	100	83	95	83	96	100	96	100	100	99	65	100	0	0	0	0	0	65	100	100	100	
*8% NaCl	1	0	44	62	0	0	71	0	0	71	78	94	80	0	0	0	0	0	0	0	0	0	0	0	
*10% NaCl	0	0	4	0	0	0	4	0	0	0	0	69	2	0	0	0	0	0	0	0	0	0	0	0	
12% NaCl	0	0	0	0	0	0	0	0	0	0	0	17	1	0	0	0	0	0	0	0	0	0	0	0	
Swarming (marine agar, 25°C) <sup>c</sup>	—	—	—	+	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—	
String test	100	100	100	80	100	80	100	80	100	100	100	91	64	100	100	0	0	0	0	0	100	100	100	100	
O129; zone of inhibition <sup>d</sup>	99	95	90	25	40	90	31	0	0	31	0	19	20	98	100	0	0	0	0	0	98	100	100	100	
Polymyxin B, % with a zone of inhibition	22	88	100	92	100	85	100	85	100	100	89	63	54	3	100	0	0	0	0	0	3	100	100	100	

<sup>a</sup>An \* indicates that the test is recommended as part of the routine set for *Vibrio* identification. 1% NaCl in parentheses indicates 1% NaCl has been added to the standard media to enhance growth; HIB, heart infusion broth; TSI, triple sugar iron agar; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.  
<sup>b</sup>The number gives the percentage positive after 48 h of incubation at 36°C (unless other conditions are indicated). Most of the positive reactions occur during the first 24 hours. NG (no growth) means that the organism does not grow, probably because the NaCl concentration is too low.  
<sup>c</sup>Symbols: +, most strains (generally about 90 to 100%) positive; —, most strains negative (generally about 0 to 10% positive).  
<sup>d</sup>Content of the disk was 10 µg.

Table 8. Key characteristics of *Vibrio cholerae* and *V. mimicus* and tests for their differentiation.<sup>a</sup>

Test or property	<i>V. cholerae</i>	<i>V. mimicus</i>
Frequency of isolation	Very common	Occasional
Properties of both species:		
Oxidase	+	+
Growth in nutrient broth with:		
No added NaCl	+	+
1% NaCl	+	+
Lysine decarboxylase	+	+
Arginine dihydrolase	–	–
Ornithine decarboxylase	+	+
Differentiation of the species:		
Sucrose fermentation	99 <sup>b</sup>	0
Lipase (corn oil)	95	10
Voges-Proskauer	95 <sup>c</sup>	0
Lactose fermentation (1–2 days)	9	74
Lactose fermentation (3–7 days)	24	56

<sup>a</sup>All data are for reactions within 2 days at 35–37°C unless otherwise specified. Symbols: +, 90 to 100% positive; –, 0 to 10% positive.

<sup>b</sup>Each number gives the percentage of positive reactions after 24 to 48 h of incubation at 35–37°C (unless another temperature or time is indicated). Most of the positive reactions occur during the first 24 h of incubation.

<sup>c</sup>Almost all strains of *V. cholerae* non-O1 and most strains of *V. cholerae* O1 currently isolated are of the eltor biogroup, which is almost always Voges-Proskauer positive; the classical biogroup is Voges-Proskauer negative.

Table 9. Differentiation and properties of the classical and eltor biogroups of *V. cholerae* serogroup O1.<sup>a</sup>

Test or property	Biogroup	
	Classical	Eltor
Frequency of isolation:		
On the Indian subcontinent	Occasional <sup>b</sup>	Common
In the rest of the world	Very rare	Common
Differential test:		
Hemolysis of red blood cells	–	+
Voges-Proskauer	–	+
Inhibition by polymyxin B (50-unit disk)	+	–
Agglutination of chicken red blood cells	–	+
Lysis by bacteriophage:		
Classical IV	+	–
FK	+	–
Eltor 5	–	+

<sup>a</sup>Symbols: +, most strains (generally about 90 to 100%) positive; –, most strains negative (generally about 0 to 10% positive).

<sup>b</sup>The classical biotype reappeared several years ago on the Indian subcontinent and has been found only in some locations.

Table 10. Antigenic serofactors of *Vibrio cholerae* O1: subtypes Ogawa, Inaba, and Hikojima.

Serotype O1 subtype	O factors present in culture	Agglutination in absorbed serum:	
		Ogawa <sup>a</sup>	Inaba <sup>a</sup>
Ogawa	A, B	+	–
Inaba	A, C	–	+
Hikojima <sup>b</sup>	A, B, C	+	+

<sup>a</sup>The specific factor sera are prepared by absorption. For example, an Ogawa antiserum is prepared by injecting an Ogawa culture, and then absorbing the resulting antiserum with an Inaba culture, which removes the antibodies to O antigen factor A, leaving antibodies to O factor B.

<sup>b</sup>Some authorities do not recognize subtype Hikojima, and report these cultures as either Inaba or Ogawa, based on which serum causes the quickest and strongest agglutination.

Table 11. Subdivision of *Vibrio cholerae* below the level of species.

*Vibrio cholerae* serogroup O1<sup>a</sup>

- Serogroup Inaba
  - Biogroup classical
  - Biogroup eltor
- Serogroup Ogawa
  - Biogroup classical
  - Biogroup eltor

*Vibrio cholerae* non-O1

- Sakazaki serotyping system: 60 O-antigen groups
- Smith serotyping system: 72 serogroups

<sup>a</sup>A third serogroup called “Hikojima” is recognized by some authors. See note on Table 10.

Table 12. Differentiation of the arginine-positive species—*Vibrio damsela*, *V. fluvialis*, and *V. furnissii*; comparison with *Aeromonas*.<sup>a</sup>

Test or property	<i>V. damsela</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>Aeromonas</i>
Growth in nutrient broth with:				
No added NaCl	–	–	–	+
1% NaCl	+	+	+	+
6% NaCl	+	+	+	–
Voges-Proskauer	+	–	–	V
Citrate, Simmons	–	+	+	V
Fermentation of:				
D-Galacturonic acid	–	+	+	–
L-Arabinose	–	+	+	V
D-Mannitol	–	+	+	+
Sucrose	–	+	+	(+)
Gas production during fermentation	V	–	+	V

<sup>a</sup>All data are for reactions within 2 days at 35–37°C unless otherwise specified. Symbols: +, 90 to 100% positive; (+), 75 to 89.9% positive; V, 25.1 to 74.9% positive; –, 0 to 10% positive.

Table 13. Differentiation of *Vibrio fluvialis* and *V. furnissii*.

Test or property	Percentage positive for <sup>a</sup>	
	<i>V. fluvialis</i>	<i>V. furnissii</i>
Simple tests		
Gas production during fermentation	0	99
Esculin hydrolysis	72	0
Carbon-source utilization, <sup>b</sup> growth on:		
Citrulline	97	4
D-Glucuronic acid	94	7
Putrescine	31	100
δ-Aminovalerate	0	63
Cellobiose	63	4
Glutaric acid	–	+

<sup>a</sup>Each number gives the percentage positive after 48 h of incubation at 35–37°C (unless other conditions are indicated). Most of the positive reactions occur during the first 24 hours.

<sup>b</sup>Data from Lee et al. 1981 except for glutaric acid, which is from Baumann et al. 1984. These carbon-source utilization tests are usually done in research laboratories rather than in public health or clinical laboratories.

Table 14. Differentiation of the arginine-negative, lysine-positive species *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. carchariae*.<sup>a</sup>

Test or property	<i>V. alginolyticus</i>	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. carchariae</i>
Voges-Proskauer	+	–	–	–
Growth in nutrient broth with:				
8% NaCl	+	(+)	–	–
10% NaCl	v	–	–	–
Fermentation of:				
Sucrose	+	–	(–)	+
Salicin	–	–	+	–
Cellobiose	–	–	+	–
Lactose	–	–	(+)	–
L-Arabinose	–	(+)	–	–
Swarming (marine agar, 25°C)	+	+	–	+
Size of zone of inhibition around:				
Colistin	Large	Large	Small	Small
Ampicillin	Small	Small	Large	Small
Carbenicillin	Small	Small	Large	Small

<sup>a</sup>All data are for reactions within 2 days at 35–37°C unless otherwise specified. Symbols: +, 90 to 100% positive; (+), 75 to 89.9% positive; (–), 10.1 to 25% positive; –, 0 to 10% positive.

simple biochemical tests given in Table 15. The biochemical test media are inoculated, read at days 1 and 2 (and up to seven days if desired). Each test result is coded as “+” or “-” for the computer, which then compares the profile of the unknown strain to all the organisms in the data base. The approach is based on the normalized likelihood calculation described by Lapage 1974. Two different programs are used: the first, named “George,” is for all species of Enterobacteriaceae and for the species of *Vibrio* that grow well at 35–37°C in media with no added salt (0.5–1.0% NaCl). A second program named “Neptune” was designed specifically to identify the halophilic species of *Vibrio* and *Photobacterium*. The biochemical tests used in this program have marine cations added (Table 15) to give a higher content of Na<sup>+</sup>, K<sup>+</sup>, and Mg<sup>++</sup>, and all incubations are done at 25°C since many of the species do not grow well at 35–37°C. Both programs are useful in comparing an unknown to a large data base of species, biogroups, and individual strains. The programs originally were run on a main-frame computer, but we have modified them to run on IBM-compatible microcomputers with DOS. They may be obtained by writing J. J. Farmer.

### Bioluminescence Test

This is a useful differential test because a few species are usually bioluminescent (*V. fischeri*, *V. logei*, *V. orientalis*, *V. splendidus* biogroup 1, *Photobacterium phosphorium*, and *P. leiognathi*), in contrast to most of the other species. Some strains of *V. harveyi* are luminescent, as are a scant few strains of *V. cholerae*. None of the members of the Enterobacteriaceae (with the lone exception of *Xenorhabdus luminescens*) and Pasteurellaceae, or of the genera *Aeromonas* and *Plesiomonas* are bioluminescent.

#### Bioluminescence Test for Vibrios

Inoculate the test strain on a medium or several media that allow good growth, such as marine agar, commercially available *Photobacterium* agar, LM medium, or trypticase soy agar (for organisms that do not require Na<sup>+</sup>), and incubate at 25°C. Also inoculate quality-control strains which are “strong” and “weak” for bioluminescence. After incubation for about 8 h and again at 16–20 h, take the plates into a room that can be totally darkened and set them on a bench top. Take in a flashlight, and turn off the overhead light. Observe the weak and strong quality-control cultures for light. Light from the strong positive culture should be visible within a few seconds, but the weak positive culture may take several minutes. Continue to observe the test culture for luminescence and record as positive or negative after 5 and again at 10 minutes. For quality control, include a strong positive such as *Vibrio fischeri* (or a similar reference culture or marine isolate); also include a weak positive such as *Xenorhabdus luminescens* or a weakly luminescent

marine strain that takes 3–5 min of dark adaptation to be seen. Test as many cultures as possible for bioluminescence at one time because this test is time consuming to set up. A photographic dark room is a good place to do the observation for light emission.

### Carbon Assimilation Tests (Baumann and Baumann, 1981)

The medium described below which is used for assimilation tests was described by McLeod (1968) to study the growth of marine bacteria on different substrates, and was subsequently used by Baumann and Baumann (1981). The carbon sources are added to the basal medium at a final concentration of 0.1 to 1% as described in “Isolation—Marine and Environmental Samples.”

#### Marine Broth

Marine broth is useful for the isolation and cultivation of marine organisms. A dehydrated powder is available from Difco (catalog no. 0791). It uses the following recipe:

Peptone (Bacto)	5 g
Yeast extract	1 g
Ferric citrate	0.1 g
Sodium chloride	19.45 g
Magnesium chloride (dried)	5.9 g
Sodium sulfate	3.24 g
Calcium chloride	1.8 g
Potassium chloride	0.55 g
Sodium bicarbonate	160 mg
Potassium bromide	80 mg
Strontium chloride	34 mg
Boric acid	22 mg
Sodium silicate	4 mg
Sodium fluoride	2.4 mg
Ammonium nitrate	1.6 mg
Disodium phosphate	8 mg
Distilled water	1 liter
Adjust to pH 7.6.	

Add 37.4 g of Difco marine broth to the distilled water and heat to boiling. Dispense into tubes or bottles and autoclave at 121°C for 15 minutes. A brown gelatinous precipitate is produced during autoclaving which settles to the bottom.

#### Marine Semisolid Medium

This is a convenient semisolid medium for maintaining stock cultures of *Vibrio*, *Photobacterium*, or other marine organisms that require added Na<sup>+</sup> and other ions for growth.

Marine broth (Difco 0791)	37.4 g
Agar	4 g
Distilled Water	1 liter

Add the marine broth to the water. Add 4 g of agar, and heat to boiling. Dispense 6 ml into 13 × 100 mm screw cap tubes. Tighten the caps and autoclave at 121°C for 15 minutes.

#### Marine Cations Supplement 1558

This medium is useful for increasing the salt content of bacteriological media to enhance the growth of marine bacteria. It contains Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>++</sup>, and Ca<sup>++</sup> at 10 times







the in-use concentration. It is our formulation which was modified from the “electrolyte supplement” of Furniss et al. (1978), and it is added in the ratio of one volume of supplement to nine volumes of medium.

Sodium chloride (NaCl)	150 g
Potassium chloride (KCl)	3.7 g
Magnesium chloride (MgCl <sub>2</sub> · 6H <sub>2</sub> O)	51 g
Calcium chloride (CaCl <sub>2</sub> · 2H <sub>2</sub> O)	7.4 g
Water	912 ml

Dissolve the ingredients in the order listed. All should dissolve readily, and a crystal-clear, colorless solution should result. The volume of the solution will expand to 1 liter after the salts dissolve. Dispense and autoclave at 121°C for 15 minutes. It should remain crystal clear after autoclaving, but a slight amount of fine precipitate may form and settle to the bottom of the container. To use this supplement in other media: aseptically add one volume of marine cations, supplement 1558 to nine volumes of the sterile medium, and mix thoroughly.

### Marine Cations Supplement 1559

This medium is the same as marine cations supplement 1558 except that it lacks the calcium chloride. It contains Na<sup>+</sup>, K<sup>+</sup>, and Mg<sup>++</sup> at 10 times the in-use concentration. This avoids precipitates, which are often produced when media containing supplement 1558 are autoclaved.

Sodium chloride (NaCl)	150 g
Potassium chloride (KCl)	3.7 g
Magnesium chloride (MgCl <sub>2</sub> · 6H <sub>2</sub> O)	51 g
Water	912 ml

Dissolve the ingredients in the order listed. All should dissolve readily, and a crystal-clear, colorless solution should result. The volume of the solution will expand to 1 liter as the salts dissolve. Dispense and autoclave at 121°C for 15 minutes. A crystal clear solution should result after autoclaving, but a slight amount of fine precipitate may form and settle to the bottom of the container.

To use this supplement in other media: aseptically add one volume of marine cation supplement 1559 to nine volumes of the sterile medium, and mix thoroughly.

### Susceptibility to the Vibriostatic Compound O129

This test of susceptibility to O129 (2,4-diamino-6,7-diisopropyl-pteridine phosphate) was originally used to differentiate cultures of *Vibrio* (usually susceptible) from *Aeromonas* (very resistant), but the test has differential value in the family Enterobacteriaceae, and can also be used to differentiate cultures of the family Pasteurellaceae from Enterobacteriaceae (Chatelain et al., 1979). Table 16 gives the susceptibility of three families.

Almost all methods for testing O129 susceptibility use paper disks that have been soaked in the compound. Originally the disks had to be prepared in the laboratory (Furniss et al., 1978), but now commercial disks are available (see below). Several different methods have been used to measure O129 susceptibility, but the method used by the Maidstone *Vibrio* Laboratory in England (Furniss et al., 1978) is used in many laboratories.

Commercial O129 disks contain 10 µg or 150 µg per disk and can be ordered from Oxoid. They should be stored in the refrigerator.

*Test method:* Grow the organism and dilute the turbidity until it is the same as a McFarland 0.5 standard. Insert

Table 16. Susceptibility of Enterobacteriaceae, Vibrionaceae, and Pasteurellaceae to the compound O129 (2,4-diamino-6,7-diisopropyl-pteridine phosphate).

Organism	O129 Susceptibility
Family Vibrionaceae	
<i>Vibrio</i> —many species	Very susceptible
<i>Vibrio</i> —some species	Somewhat susceptible
<i>Aeromonas</i>	Very resistant
Family Enterobacteriaceae	
<i>Edwardsiella</i>	Susceptible
Other genera	Resistant
Family Pasteurellaceae	
<i>Pasteurella</i>	Susceptible
<i>Actinobacillus</i>	Susceptible

a cotton swab into the liquid, then wring out as much as possible on the side of the tube. Streak the swab onto a nutrient agar \* or trypticase soy agar (used in our laboratory) plate in three directions. Apply 10- and 150-µg O129 disks and incubate overnight. Observe for a zone of inhibition and record the zone size to the nearest mm. Interpret as follows: sensitive, a zone around each disk; partially sensitive, a zone around the 150-µg disk but no zone around the 10-µg disk; resistant, no zone around either disk.

### Salt (Na<sup>+</sup>) Requirement and Tolerance Tests

These are useful tests for differentiating species of *Vibrio* and *Photobacterium*. Growth in 0% NaCl is an important characteristic that differentiates *Vibrio cholerae* and *V. mimicus* from the halophilic *Vibrio* species. This test has been done by many different methods, which helps to explain why published results vary among laboratories. We use the following procedure in our laboratory:

#### Salt Requirement and Tolerance Test

Grow the organisms on marine agar for 18–24 hours at 36°C (if it does not grow at 36°C, use 25°C; if it does not grow at 25°C, use 15°C). Remove some growth with a cotton swab and suspend it in nutrient broth (no added NaCl) until the turbidity is the same as a MacFarland 0.5 standard. A recipe for nutrient broth + 0.1% NaCl is given below. Immediately inoculate each of the media listed below with 0.02–0.03 ml (one drop from a disposable pasteur pipette). Incubate overnight and read for visible growth.

##### *Salt (Na<sup>+</sup>) requirement media:*

Nutrient broth + 0% NaCl  
 Nutrient broth + 0.1% NaCl  
 Nutrient broth + 0.2% NaCl  
 Nutrient broth + 0.3% NaCl  
 Nutrient broth + 0.4% NaCl  
 Nutrient broth + 0.5% NaCl  
 Nutrient broth + 1% NaCl  
 Nutrient broth + 2% NaCl  
 Marine broth

##### *Salt tolerance media:*

Nutrient broth + 6% NaCl

Nutrient broth + 8% NaCl  
 Nutrient broth + 10% NaCl  
 Nutrient broth + 12% NaCl

Interpret as follows: If the organism has no Na<sup>+</sup> requirement (or a very small one) it will grow in nutrient broth plus 0% NaCl. If it has a Na<sup>+</sup> requirement, it will not grow in 0% NaCl, but will grow with the added NaCl or in the marine broth. For each organism the minimum and maximum concentration of NaCl can be determined.

#### Nutrient Broth + 0.1% NaCl

This is a formula for one of the media mentioned above. It is essential to use a commercial formulation for nutrient broth (Difco, BBL, etc.) that does not contain NaCl; some formulas (for example, Oxoid) have NaCl as an ingredient. Nutrient broth with other concentrations of NaCl are prepared similarly.

Nutrient broth (Difco or BBL)	8 g
Sodium chloride solution, 10%	10 ml
Water	990 ml

Dissolve the nutrient broth in the water and add the 10% sodium chloride solution. Dispense 10 ml into screw-cap test tubes, replace the caps and *tighten them* to prevent evaporation and a resulting change in NaCl concentration. Autoclave at 121°C for 15 minutes. Allow the tubes to cool at room temperature, check each cap to be sure it is tight, discard any tubes that have a loose cap or obvious loss of liquid. It is convenient to dispense the media for salt requirement and tolerance into color-coded tubes. This helps to avoid errors as they are being read.

#### String Test

The string test has some differential value because many *Vibrio* species (particularly *V. cholerae*) are string-test positive, but many other species, including *Aeromonas*, are negative. Sodium deoxycholate is a detergent that lyses Gram-negative organisms. When cells are lysed, DNA is released into the suspending medium, making it very viscous and able to form “strings of DNA” when touched with a loop that is raised from the surface of the liquid.

Grow the organisms on trypticase soy agar (or marine agar for halophilic species). With a loop, remove some of the growth and make a heavy suspension in one drop (0.1 ml) of a 0.5% sodium deoxycholate solution. Every 10 seconds or so, raise the loop to see if a “string of DNA” has been formed. Interpretation:

Positive result: The solution becomes viscous and a string of DNA is obvious within 60 seconds. (Positive string test = *Vibrio cholerae*.)

Negative result: No string of DNA formed within 60 seconds. (Negative string test = *Aeromonas hydrophila*.)

#### Sodium Deoxycholate Solution, 0.5%

This reagent is used as the lysing solution for the string test.

Deoxycholic acid, sodium salt (Sigma D6750)	5 g
Water	1 liter

Slowly add the white powder to the water as it is being stirred on a magnetic stirrer. A crystal-clear, colorless solution results.

#### Serotyping

This technique is usually done by reference or research laboratories rather than by clinical laboratories, and some of the typing schemes that have been described are discussed under the specific organisms. One exception is the use of *V. cholerae* O1 antisera, which can be used in clinical laboratories to test a strain of *V. cholerae* to determine whether it is O1-positive or negative. Simonson and Siebeling (1988) described the use of latex agglutination for the routine identification of several of the *Vibrio* species. Antisera are made to the flagella which is usually species specific, absorbed to latex particles or *Staphylococcus aureus* cells, and then used in a simple slide agglutination test.

#### Antibiotic Susceptibility

The species of *Vibrio* important in clinical microbiology usually grow well on Mueller-Hinton agar, which is used in the disk susceptibility test. Although Mueller-Hinton agar contains no added NaCl, it contains a hydrochloric acid hydrolysate of casein, which apparently has been neutralized with NaOH and has enough NaCl to allow good growth of the halophilic species of *Vibrio*. However, some of the environmental marine vibrios grow poorly on Mueller-Hinton, presumably because of their higher requirement for Na<sup>+</sup>. Broth dilution susceptibility tests in Mueller-Hinton broth can be done without modification for most *Vibrio* species because the broth contains sufficient Na<sup>+</sup> (Hollis et al., 1976).

Antibiotic resistance is rare in *Vibrio* compared with Enterobacteriaceae. Table 17 summarizes the results for the strains we have tested. In most cases, resistance appears to be intrinsic to the species rather than acquired through plasmid transfer or through antibiotic exposure. The one exception to this generalization is the antibiotic resistance found in some outbreaks of *V. cholerae*, which have become resistant through the acquisition of R factors. In the United States, strains of *V. cholerae* and other *Vibrio* species have rarely had this type of resistance. Resistance to polymyxin antibiotics (polymyxin B and colistin) can be useful in spotting a culture of the eltor biogroup of *V. cholerae*, or in spotting a culture of *V. vulnificus*. Most other *Vibrio* species are more susceptible.

#### Identification—Marine And Environmental Isolates

The methods that have been used to identify isolates of *Vibrio* and *Photobacterium* isolated

Table 17. Antibiotic susceptibility (Kirby-Bauer disk method) of 1,025 strains of the *Vibrio* species that occur in human clinical specimens.<sup>a</sup>

Antibiotic (zone size range) <sup>b</sup>	Percentage of strains susceptible (number of strains studied)												
	<i>V. cholerae</i> (480)	<i>V. mimicus</i> (75)	<i>V. metschnikovii</i> (22)	<i>V. cincinnatiensis</i> (14)	<i>V. holisae</i> (34)	<i>V. damsela</i> (21)	<i>V. fluvialis</i> (25)	<i>V. furnissii</i> (9)	<i>V. alginolyticus</i> (69)	<i>V. parahaemolyticus</i> (144)	<i>V. vulnificus</i> (130)	<i>V. carchariae</i> (2)	
Penicillin G (12–21)	2	3	9	0	97	0	0	0	0	0	2	0	
Ampicillin (12–13)	87	97	31	36	100	52	32	11	0	12	99	0	
Carbenicillin (18–22)	64	8	27	7	100	14	16	0	0	1	54	0	
Cephalothin (15–17)	98	100	100	100	100	76	40	0	32	17	65	100	
Colistin (9–10)	4	61	91	93	100	76	100	100	25	11	2	0	
Tetracycline (15–18)	98	100	73	93	97	86	88	89	94	98	99	100	
Sulfadiazine (13–16)	26	17	5	36	56	71	36	11	16	3	28	50	
Chloramphenicol (13–17)	99	100	100	100	100	10	88	100	100	100	100	100	
Streptomycin (12–14)	60	61	32	86	100	24	84	100	54	17	42	50	
Kanamycin (14–17)	92	89	14	79	100	43	88	100	62	37	53	100	
Gentamicin (13–14)	98	99	100	100	100	100	100	100	100	97	100	100	
Nalidixic acid (14–18)	99	99	100	100	100	100	100	100	97	99	99	100	

<sup>a</sup>Studied at the CDC *Vibrio* Laboratory and done on Mueller-Hinton agar (with no added NaCl) at 35–37°C.

<sup>b</sup>The numbers in parentheses give the zone size range for the category “intermediate.” For example, “(12–21)” means that resistant strains have 6– to 11–mm zones, strains of intermediate susceptibility have zones that are 12– to 21–mm, and susceptible strains have zones of 22 mm or larger. These particular break points are the ones established in the early 1970s for each antibiotic, and they have been used in our laboratory for over 15 years for taxonomic studies. They may differ slightly from current break points.

from the environment are quite varied because these organisms have been studied in different laboratories, and each has tended to use their own methods. The methods described in this section are taken from Baumann and Baumann (1981) and have proved useful in the research laboratory setting.

### Cellular Morphology

**CELL SIZE AND SHAPE** When examined during the exponential phase of growth in yeast extract broth (YEB) or other relatively simple media, the cells of most *Vibrio* and *Photobacterium* generally appear to be regular, straight or curved rods (Figs. 8, 9, 14, and 15). Many species give rise to involution forms in early stationary phase, a tendency particularly noticeable in some strains of *Photobacterium*. For this reason, it is important to examine the morphology of marine bacteria in the exponential phase of growth in liquid medium. Many of these involution forms are spherical and have been called "spheroplasts" or "round bodies" (Felter et al., 1969; Levin and Vaughn, 1968). These designations are unfortunate since they imply a regularity in the frequently bizarre shapes observed in old cultures (Felter et al., 1969; Kennedy et al., 1970). In *Desulfovibrio aestuarii* and *Nitrospina gracilis*, the formation of "spheroplasts" and "round bod-

ies" was accompanied by a loss of viability (Levin and Vaughn, 1968; Watson and Waterbury, 1971). Baker and Park (1975) have shown that the formation of these structures in stationary phase cultures of a *Vibrio* species correlates with a decrease in the amount of peptidoglycan and loss of viability. The early stages in the formation of involution forms in marine enterobacteria observed by phase contrast microscopy and electron microscopy (Baumann and Baumann, 1981) resemble the  $K^+$ -depleted, plasmolyzed cells of *Alteromonas haloplanktis*. Since the intracellular  $K^+$  content of this organism is involved in the maintenance of turgor and since energy is required for  $K^+$  accumulation and maintenance in the cell (Thompson and MacLeod, 1973), it is possible that one of the early manifestations of cell death is a loss of the ability to maintain a high intracellular  $K^+$  concentration leading to plasmolysis and resulting in structures which subsequently become "spheroplasts," "round bodies," or other involution forms. Colwell (1973) has stated that the formation of "round bodies" is a characteristic of the genus *Vibrio*, but these involution forms are observed in a wide variety of organisms, including strict aerobes of marine origin (Baumann et al., 1972), species of *Photobacterium*, and the nitrifying bacterium *Nitrospina gracilis* (Baumann and Baumann, 1981).

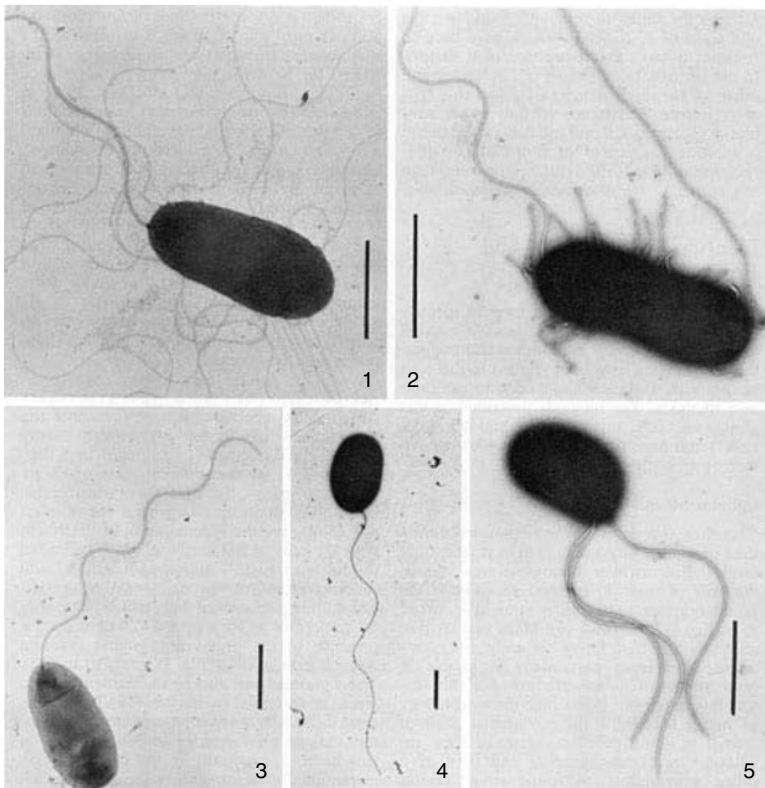


Fig. 14. Different sizes and shapes of *Vibrio* and *Photobacterium* strains seen in electron micrographs. (Parts 1-3 from Allen and Baumann, 1971. Parts 4 and 5 courtesy of R. D. Allen.)

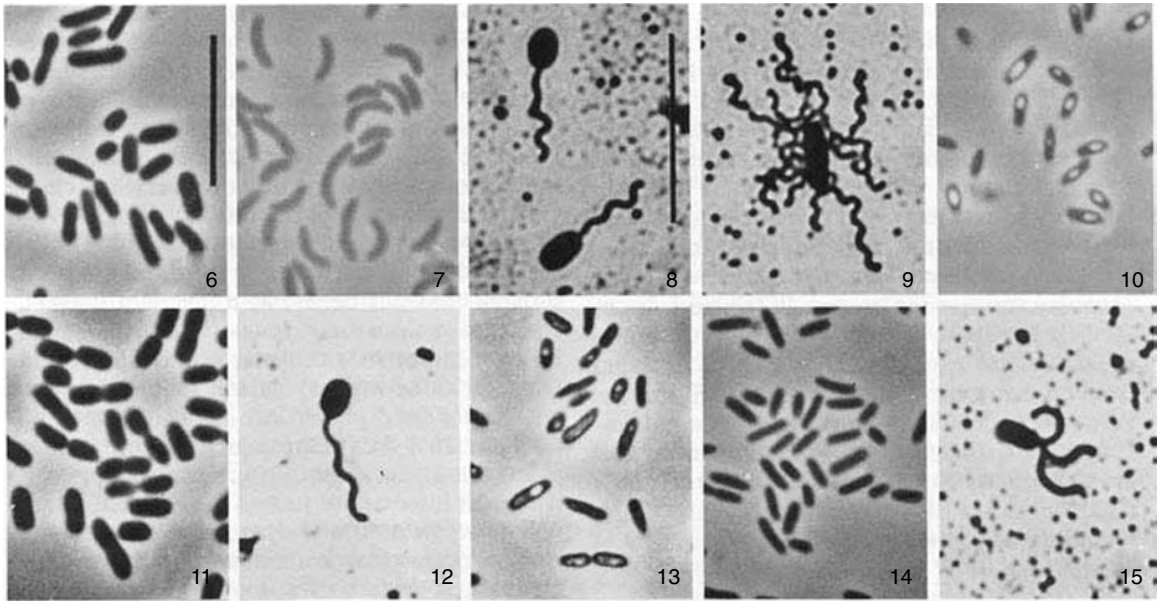


Fig. 15. Different sizes and shapes of *Vibrio* and *Photobacterium* strains seen in phase contrast micrographs and flagella stains. (Parts 6, 7, 10, and 15 from Baumann et al., 1971a; Parts 8 and 9 courtesy of P. Baumann.)

**ACCUMULATION OF POLY- $\beta$ -HYDROXYBUTYRATE (PHB)** Many species of marine eubacteria can accumulate PHB as an intracellular reserve product. In the scheme of Baumann and Baumann (1981) for the differentiation of *Photobacterium* species, this trait was of considerable taxonomic importance. In general, the simplest way to observe the accumulation of PHB is to grow the cells in BM medium which is limited for nitrogen (0.02% ammonium sulfate) and contains excess (0.4%) DL- $\beta$ -hydroxybutyrate. The culture is examined daily with a phase contrast microscope, for a period of 4 days, for the presence of the bright intracellular granules characteristic of PHB (Fig. 15, parts 10 and 13). These granules can also be stained with Sudan black, as described by Burdon (1946). In some cases difficulties are encountered since the granules may be small and, in addition, some marine bacteria, especially in old cultures, have involution forms containing inclusions which may be confused with PHB. In a research setting, PHB can also be identified chemically by alkaline hypochlorite digestion of the cells followed by the solubilization of the PHB in chloroform and precipitation with acetone (Williamson and Wilkinson, 1958). A quantitative estimation of the extracted PHB can be made by spectrophotometric methods.

**FLAGELLA STAINS** Some strains have tubular projections (Fig. 14), part which are evaginations of the outer membrane of the cell wall (Allen and Baumann, 1971; Baumann et al., 1972) and these might be mistaken for flagella. However, these evaginations are usually readily distin-

guishable from flagella since they are straight and lack the wave form characteristic of flagella. The flagellins of the polar and peritrichous flagella in the same strain differ in their amino acid composition as well as in their immunological properties (Shinoda et al., 1974a, 1974b, 1976). Genetic studies have shown that the peritrichous flagella are essential for swarming on solid media while the polar flagella are necessary for motility in liquid media (Shinoda and Okamoto, 1977).

### Carbon Sources Used For Growth

Baumann and Baumann (1981) indicate that the *Vibrio* and *Photobacterium* ferment only a few carbohydrates. Thus, the fermentation tests that have been extremely useful in the identification of Enterobacteriaceae have been of more limited value for the vibrios. Baumann and Baumann (1981) studied many different carbon compounds and found considerable variation among the species in their utilization pattern. Different species of *Vibrio* and *Photobacterium* vibrios use a range of from 14 to 67 organic compounds as sole sources of carbon and energy; these include pentoses, hexoses, disaccharides, sugar acids, sugar alcohols, 2-carbon to 10-carbon monocarboxylic fatty acids, tricarboxylic acid cycle intermediates, and amino acids. Species utilizing aromatic compounds degrade the intermediate protocatechuate by means of a "meta" cleavage. None of the species utilize cellulose, formate, dicarboxylic acids with 6 to 10 carbon atoms, L-isoleucine, L-valine, L-lysine, L-tryptophan, purines, pyrimidines, or *n*-hexadecane.

## Extracellular Enzymes

The production of extracellular chitinase, alginase, amylase, gelatinase, and lipase is determined on the appropriate solid medium inoculated as a spot or streak. A positive reaction is observed as a zone of hydrolysis beyond the limits of growth. Several strains can be tested on one plate, or they can be tested in tubes. Chitinase production is tested on yeast extract agar (YEA) plates overlaid with 10–15 ml of modified YEA containing about 5 g colloidal chitin per liter (prepared as described by Berger and Reynolds, 1958) and 2.5 g yeast extract per liter. To test for alginase activity, colloidal chitin is replaced by 20 g of sodium alginate per liter. An overlay method is not needed to detect the presence of amylase, gelatinase, and lipase. To test for these extracellular enzymes, YEA is supplemented with 2 g starch, 50 g gelatin, or 10 ml polyethylene sorbitan monooleate (Tween-80) per liter, respectively. The hydrolysis of chitin or alginate results in a zone of clearing, whereas lipase activity on Tween-80 plates is detected by the appearance of a precipitate of calcium oleate; all three are observed for a period of 7 days. After 48 h of incubation, the starch plates are flooded with Lugol's iodine solution (Stanier et al., 1966) and the gelatin plates with acidic mercuric chloride in order to visualize the unhydrolyzed polymer.

## *Vibrio* Species of Medical Importance

The following sections give a summary of the information available on each of the 12 species of *Vibrios* that are causes of human disease.

### *Vibrio cholerae*

*V. cholerae* is by far the most important species in the genus *Vibrio*. It has caused many epidemics of cholera and millions of deaths. Once, it was one of the most feared bacterial pathogens in the world (Pollitzer, 1959).

In the mid-1930s, it was recognized that the vast majority of vibrios isolated from cholera cases agglutinated in a single antiserum (Pollitzer, 1959). Agglutination in this antiserum (later called *V. cholerae* O1 antiserum) became the main criterion for identifying a culture as *V. cholerae*. Organisms that did not agglutinate in this serum were given vernacular names such as “nonagglutinating vibrios,” “NAGs,” or “non-cholera vibrios.” These included some organisms known today as *Vibrio cholerae* non-O1, *V. parahaemolyticus*, other *Vibrio* species, and even species of *Aeromonas* or *Plesiomonas*. It was soon

recognized that some of the “NAGs” were identical with *V. cholerae* except for their agglutination in O1 antiserum (Sakazaki and Balows, 1981). However, it has only been in the last few years that these have been classified in the species *V. cholerae*. Today it is accepted that there are two main groups of *V. cholerae* strains—serogroups “O1” and “non-O1.” It is usually convenient to discuss these separately. *V. cholerae* O1 is usually isolated from cholera cases, where it often produces severe watery diarrhea through the action of cholera toxin. *V. cholerae* non-O1 can cause a cholera-like illness but it can also cause a much wider spectrum of disease (Morris et al., 1981), including extraintestinal infections (Hughes et al., 1978).

### *Vibrio cholerae* Serogroup O1

As discussed above this group of *V. cholerae* strains agglutinate in O1 serum, and include serological subtypes Ogawa and Inaba (and Hikojima) (Donovan and Furniss, 1982), and include two biogroups, classical and eltor.

**HISTORY** The cholera bacillus was isolated by Koch in 1884, so bacteriological confirmation of cholera was possible only after this date. Cholera, or Asiatic cholera as it was often called in early writings, has probably existed in India since ancient times. It may have been found outside of India before 1800, but not in large epidemics. The spread of Asiatic cholera has come in seven large pandemics. The first pandemic began in 1816–1817; six other pandemics followed beginning respectively in 1829, 1852, 1863, 1881, 1889, and 1961 (Pollitzer, 1959), all except the last caused by the classical biogroup. Although the seventh pandemic of 1961 was caused by the eltor biogroup, the classical biogroup has displaced the eltor biogroup in some parts of the Indian subcontinent.

**HABITATS** *V. cholerae* O1 is found in the human intestinal tract where it can cause a wide spectrum of disease ranging from mild diarrhea to a fatal cholera. The organism is rarely found in other environments unless they have been contaminated with feces from infected individuals. Recent indigenous cases of toxigenic *V. cholerae* O1 infection in the United States and Australia have suggested the possibility of an environmental reservoir.

**SYMPTOMS** In an area where cholera is endemic, many individuals who ingest *V. cholerae* may have either a mild diarrhea or no symptoms at all (asymptomatic colonization of the intestine with recovery of the organism from formed

stool). This pattern of mild disease was much more common in the seventh (1961) cholera pandemic caused by the eltor biogroup of *V. cholerae*. At the other extreme, some individuals develop an acute diarrhea with constant purging that has been called “cholera gravis.” In severe cholera there is massive diarrhea, with large volumes of rice-water stool (clear fluid with flecks of mucus) passed painlessly. The amount of fluid passed can be a liter or more per hour. In 4–6 days this would amount to over twice the body weight. There is usually vomiting and little desire to eat. If untreated, there will be prostration with symptoms of severe dehydration, electrolyte imbalance, painful muscle cramps, watery eyes, loss of skin elasticity and anuria (absence of urine excretion). Death can occur very quickly after onset of symptoms because of the severe dehydration.

**TREATMENT** Treatment for the most severe cases of cholera is intravenous therapy with large volumes of a simple balanced salts solution which restores water and electrolyte balance and prevents acidosis. Cases with mild or moderate illness are often treated with oral electrolyte solutions. Tetracycline therapy reduces the period of excretion but is not a substitute for rehydration.

Milder forms of diarrhea due to *V. cholerae* O1 are more difficult to distinguish from other mild diarrheas. They can last from 1–5 days with the passage of several liquid stools per day. Cramping and vomiting can also occur. The role of *V. cholerae* O1 can only be shown by isolating and identifying the causative organism. In patients with the eltor biogroup of *V. cholerae* O1, this milder disease is seven times more common than severe cholera.

**PATHOGENESIS** The pathogenesis of diarrhea due to *V. cholerae* O1 is well understood. The organism is ingested and some cells survive the acid pH of the stomach and pass into the small intestine. The organism colonizes the small intestine and begins to grow and produce cholera toxin. Cholera toxin, which has a molecular weight of 84,000, is composed of an A subunit (molecular weight 21,000), A2 subunit (molecular weight 7,000), and five B subunits (molecular weight 10,000 each). The B subunit of intact cholera toxin attaches to a specific receptor, ganglioside G1, on the cell membrane of cells in the intestine. The A1 subunit of cholera toxin then activates the enzyme adenylate cyclase of the host which then increases the level of cyclic AMP, leading to the hypersecretion of salt and water. The net result is the massive outpouring of liquid stool and resulting dehydration which is typical of cholera.

**SOURCES** *V. cholerae* O1 is an intestinal pathogen, and the vast majority of strains come from human feces (Tables 2 and 3). Only a few human isolates are extraintestinal. *V. cholerae* O1 can also be isolated from the environment and from other animals that come in contact with feces of cholera cases. There are occasionally reports of *V. cholerae* O1 from environmental sources that are unlikely to have come from cholera cases. Most of these strains do not produce cholera toxin, and may represent strains of *V. cholerae* from an evolutionary line unrelated to the one that causes pandemic cholera. They are more similar to the *V. cholerae* non-O1 strains in their clinical and public health importance.

**GEOGRAPHICAL DISTRIBUTION** The World Health Organization (WHO) regularly reports the world-wide distribution of reported cholera cases. In its survey for 1989, 48,403 cases were reported from 35 countries, but some governments probably do not report cases for political and economic reasons. Almost all of the cases were in Africa (35,606) and Asia (12,785).

The history of cholera (defined to be the disease caused by toxin-producing *V. cholerae* O1) in the United States is quite interesting (Blake et al., 1980a). The last case of cholera in the United States from a pandemic occurred before 1900. Then, unexpectedly, there was an isolated case from Texas in 1973. This was followed by an outbreak in Louisiana in 1978, two sporadic cases in Texas in 1982, and an outbreak on an oil-drilling platform on a bayou near the Texas coast in 1982, with sporadic cases since. These strains have been thoroughly studied and have the following properties in common: serogroup Inaba (see Table 10), cholera toxin positive, strongly hemolytic, and a unique bacteriophage lysis pattern. When compared to other strains from the seventh pandemic of cholera, the American strains are quite different, suggesting that this is a different clone. Apparently, a similar situation is occurring in Australia, which has its own unique clone(s).

**ISOLATION** In rice-water stools from cholera cases, *V. cholerae* is usually present essentially as a pure culture and in very high numbers ( $10^6$  to  $10^8$  organisms per ml stool). Isolation will present no problem in these cases (Figs. 6, 7, 8, and 11). In formed feces, the organism will probably be present in much lower numbers and normal enteric flora will also be present. Enrichment in alkaline peptone water and plating on a highly selective medium will yield some additional positive cultures in this situation (Balows et al., 1971).

**IDENTIFICATION** In countries where cholera is common there is no need to do a large number

of biochemical tests to confirm a culture as *V. cholerae*. However, complete biochemical testing should be done in countries where cholera is rare (Tables 6 to 11). The test for the Na<sup>+</sup> requirement differentiates *V. cholerae* from the halophilic *Vibrio* species (Table 6). Sucrose fermentation differentiates *V. cholerae* from *V. mimicus* (Table 8). The decarboxylase pattern of arginine negative, lysine positive, and ornithine positive differentiates (with one exception) *V. cholerae* from the oxidase-positive fermentative species in *Aeromonas* and *Plesiomonas*.

**ANTIBIOTIC SUSCEPTIBILITY** Resistance is rare in *V. cholerae*. Most strains are susceptible to tetracycline, the drug of choice. Occasionally a strain of *V. cholerae* becomes resistant and can spread. This usually happens in cholera-endemic areas where there is more chance for a strain to acquire an antibiotic resistance plasmid. Susceptibility to the polymyxin group of antibiotics is a property used to differentiate the classical and eltor biogroups (Table 9).

### *Vibrio cholerae* Non-O1

This group of organisms is strikingly similar to *V. cholerae*, but they do not agglutinate in O1 antiserum. They can cause a cholera-like disease, but are usually isolated from patients with mild diarrhea and from extraintestinal infection and the environment, which is a very important reservoir. These organisms were originally not classified in the species *V. cholerae* but were reported as “nonagglutinating vibrios,” “NAGs,” or “non-cholera vibrios.” Now, however, they are classified as *V. cholerae* non-O1, much data are appearing on their ecology (Colwell, 1984) and role in human disease (Hughes et al., 1978; Morris et al., 1981).

**HABITATS** Strains of *V. cholerae* non-O1 have been isolated from patients with severe dehydrating (cholera-like) gastroenteritis. Other strains have been isolated from patients with mild diarrhea. Unlike *V. cholerae* O1, the non-O1 strains have also been found in patients with diarrhea and fever whose feces have contained blood and/or mucus.

Although the pathogenic process is not as well known as is that of *V. cholerae* O1, three possible mechanisms have been postulated: 1) production of cholera (or cholera-like?) toxin; 2) production of a heat-stable enterotoxin (positive in the infant mouse assay used for *Escherichia coli* heat-stable enterotoxin); or 3) invasive disease (positive response to whole cells but not to cell filtrates in the ligated rabbit ileal loop or infant mouse assay). However, some strains of *V. cholerae* non-O1 have no positive response in any of

the above assays, so the correlation of the possible virulence factors and the different types of gastroenteritis-invasive diarrhea need further investigation.

**EXTRAIESTINAL INFECTIONS** *V. cholerae* non-O1 has a wider spectrum of disease than the *V. cholerae* O1. Non-O1 strains have been isolated from patients with septicemia who have cirrhosis and/or other underlying disease. In our collection (Table 3), there were 42 blood isolates, compared with only one blood isolate of *V. cholerae* O1. The non-O1 strains were also isolated from ears, wounds, respiratory tract, and urine. There were 52 isolates from these sources compared with only two for *V. cholerae* O1 (Table 3).

**GEOGRAPHICAL DISTRIBUTION** *V. cholerae* non-O1 strains have been isolated worldwide both from patients and the environment. In the United States many are found in sporadic cases of diarrhea. Most of these have occurred after eating raw oysters. In the United States less than 5% of the isolates have been positive for cholera toxin; this is in contrast to the situation in Bangladesh where about one-third of the isolates have been positive (Blake, 1981).

**ISOLATION AND IDENTIFICATION** The discussion for *V. cholerae* O1 also applies to the non-O1 strain. The essential difference is that the O1 strains agglutinate in antiserum to *V. cholerae* O1 but the non-O1 strains do not. The biochemical reactions of both *V. cholerae* groups are almost identical.

**SEROTYPING** Strains of *V. cholerae* non-O1 can be further differentiated by serotyping by one of several systems (Brenner et al., 1982; Smith, 1979). In a system described by Sakazaki and Shimada (1977), antisera are made against heated cells, and the O antigen is determined by agglutination. There are over 60 different O antigens in this schema. In a system described by Smith (Smith, 1979), antisera are made against unheated cells, and the “Smith type” is determined by slide agglutination of living cultures. There are over 72 different types in the Smith schema. Serological typing of non-O1 *V. cholerae* should be done only to answer specific epidemiological questions, and is done by only a few reference laboratories. An international working group has been proposed to study the two systems and make recommendations for standardization.

### *Vibrio mimicus*

*Vibrio mimicus* is a species of *Vibrio* (Davis et al., 1981) that apparently causes diarrhea, usu-



ally after the consumption of uncooked seafood, particularly raw oysters (Shandera et al., 1983). *V. mimicus* has been isolated from many countries (Canada, Mexico, Philippines, New Zealand, Guam, Bangladesh) which suggests a worldwide distribution in countries situated along an ocean. The isolation of *V. mimicus* from water, oysters, and shrimp, and its distribution in coastal areas indicate that its ecology may be similar to the ecology of *V. cholerae* non-O1.

**HISTORY** There have been a number of reports of *V. cholerae* strains with atypical biochemical reactions. Traditionally they have been reported with designations such as “*V. cholerae*—lysine-decarboxylase negative,” “*V. cholerae*—mannitol negative,” or “*V. cholerae*—sucrose negative.” Davis et al. (1981) studied representative strains from six of these unusual biogroups by both DNA hybridization and phenotypic analysis. Five of the six groups were highly related to *V. cholerae* by DNA hybridization. These had been identified correctly as “atypical strains of *V. cholerae*.” However, the group of sucrose-negative strains was only 24–54% related to *V. cholerae*. Based on this low relatedness and on their phenotypic differences, Davis et al. (1981) proposed a new species, *Vibrio mimicus*. The name “mimicus” refers to the fact that the strains “mimic” *V. cholerae*.

**HABITATS** *V. mimicus* has been isolated from diarrhea cases, extraintestinal infections, and the environment. Based on the information submitted with cultures, Davis et al. (1981) suggested that *V. mimicus* may be a new cause of diarrhea, probably linked to eating shellfish. Three strains of *V. mimicus* were positive for heat-stable enterotoxin in the infant mouse assay and five were positive for heat-labile enterotoxin in the Y-1 adrenal cell assay or in the ELISA assay. However, other strains from diarrhea cases were negative in the assays. The enterotoxin data were very similar to those for *V. cholerae* non-O1 strains in that some produced heat-labile or heat-stable enterotoxin, but most strains from patients with diarrhea produce neither toxin.

**ISOLATION AND IDENTIFICATION** Most of the *V. mimicus* strains have been isolated during a search for *V. cholerae* and other *Vibrio* species. *V. mimicus* grows well on TCBS agar and forms green (sucrose-negative) colonies (Table 4). *V. mimicus* grows well on the usual enteric media and in nutrient broth with no added NaCl. Isolation and identification should pose no special problems.

**BIOCHEMICAL REACTIONS** Tables 6 to 8 give the reactions of *V. mimicus* and compare them with

those of *V. cholerae*. These two species are very similar in most of the tests normally needed for identification; however, Table 8 gives reactions which are useful for differentiation. *V. mimicus* is negative for lipase production and sucrose fermentation. *V. cholerae* usually has the opposite pattern, although a few strains are lipase negative. *V. mimicus* resembles the “classical biogroup” of *V. cholerae* because it is Voges-Proskauer negative and susceptible to polymyxin. The “eltor biogroup” of *V. cholerae* is usually Voges-Proskauer positive and resistant to polymyxin. *V. mimicus* grows in nutrient broth without added NaCl, a reaction which differentiates it, along with *V. cholerae*, from the halophilic *Vibrio* species that require higher NaCl concentration for growth.

### *Vibrio parahaemolyticus*

*V. parahaemolyticus* has been known as a cause of acute gastroenteritis since 1950 (Zen-Yoji et al., 1965). Foodborne outbreaks and sporadic cases occur worldwide and are usually associated with the consumption of contaminated seafood. Two books (Fujino et al., 1974; Miwatani and Takeda, 1976) and a review (Joseph et al., 1982) summarize much of the information known about this organism.

**HISTORY** On 20 and 21 October 1950, there was an outbreak of food poisoning in Osaka, Japan, involving 272 patients with acute gastroenteritis and 20 deaths. This led to an extensive investigation and eventually an organism was isolated which was shown to be the etiological agent. The fascinating account of this discovery can be found in Miwatani and Takeda (1976, p. 1–5). The organism was studied and named as a new species, *Pasteurella parahaemolytica*. Its halophilic nature was not discovered until 1955 (Miwatani and Takeda, 1976), when it was reclassified as a halophilic *Vibrio* species, *V. parahaemolyticus*.

**SYMPTOMS** *V. parahaemolyticus* causes gastroenteritis with nausea, vomiting, abdominal cramps, low-grade fever, and chills (Barker and Gangarosa, 1974). The diarrhea is usually watery but can sometimes be bloody. The disease is usually mild and self-limiting but can be fatal (a 7% fatality rate was reported in the first outbreak). There is a good correlation between pathogenicity and a positive Kanagawa test (a test which measures the ability of the strain to produce a hemolysin for human red blood cells when grown on a special medium; Ljungh and Wadstrom, 1983). About 96% of the *V. parahaemolyticus* strains from well-documented cases of human gastroenteritis are Kanagawa positive,

but only about 1% of the strains isolated from the environment are positive. Although there is excellent correlation between this hemolysin and human disease, the mechanisms of pathogenesis are still unclear, since other toxins or virulence factors may be involved. Rehydration is usually the only treatment needed, but in some severe cases the patient will require hospital admission. Antimicrobial therapy may be beneficial, and tetracycline appears to be the drug of choice.

Outbreaks of gastroenteritis due to *V. parahaemolyticus* occur worldwide, but are not common in the United States. In Japan, *V. parahaemolyticus* is an extremely important diarrheal agent, and causes 50–70% of the cases of food-borne enteritis (Sakazaki and Balows, 1981). All of the outbreaks were associated with seafood either directly or indirectly. Direct infection comes from the ingestion of raw fish or shellfish which are contaminated with the organism. This mechanism of transmission is apparently quite common in Japan because of the national custom of eating raw fish (Sakazaki and Balows, 1981). Contamination after cooking apparently is the mechanism of indirect infections.

**ISOLATION** Much has been written about the isolation of *V. parahaemolyticus*. The fact that it was not detected as an important cause of gastroenteritis until 1950 indicates that isolation with the usual enteric media can pose problems. Oxidase testing of colonies on a nonselective medium such as blood agar should provide a high isolation rate. A plate of TCBS medium (Table 4) can be included in those geographical areas where *V. parahaemolyticus* is most common. The isolation methods designed for *V. cholerae* are also efficient for *V. parahaemolyticus*. *V. parahaemolyticus* grows well on TCBS agar as green colonies 2 to 3 mm in diameter.

**IDENTIFICATION** *V. parahaemolyticus* is one of the halophilic *Vibrio* species (Baumann and Baumann, 1973; Twedt et al., 1969) belonging in the lysine-positive, arginine-negative group (Table 6). It is negative for the Voges-Proskauer test and for fermentation of lactose and salicin. These and other tests are useful in differentiation (see Tables 6, 7, and 14).

**UREA-POSITIVE STRAINS** Initially, all strains of *V. parahaemolyticus* were reported to be negative for urea hydrolysis. Sakazaki and Balows reported that none of their 2,354 strains were urea positive (Sakazaki and Balows, 1981). However, in the last decade we have received many strains which were otherwise typical of *V. parahaemolyticus* but were urea-positive. These urea-positive strains were confirmed by DNA hybridization as *V. parahaemolyticus* (Brenner et

al., 1983a). In our laboratory the number of urea-positive strains has increased significantly: 1977, 17% urea-positive; 1978, 14% positive; 1979, 36% positive; 1980, 12% positive; 1981, 47% positive; 1982, 73% positive; and 1983, 83% positive, etc. This sample is biased because laboratories refer atypical strains rather than typical ones. However, in recent years, 50% or more of *V. parahaemolyticus* strains from California and from outbreaks investigated by CDC have also been urea-positive. These samples are less biased, and show that the increase in urea-positive strains is real.

**SEROTYPING** A serological typing schema for *V. parahaemolyticus* includes 11 numbered O antigens and 55 numbered K antigens. The O antigen is first determined by slide agglutination with an autoclaved antigen. The K antigen is then determined by slide agglutination with an unheated suspension. A complete set of O and K antisera (individuals and pools) is produced commercially by Toshiba Kagaku Kogyo Co., Inc., Maruishi Bldg. 2, 1-Chrome Kanda-Kajicho, Chiyode-KU, Tokyo, Japan. It may still be available in the United States from Nichimen Company, Inc., 1185 Avenue of the Americas, New York, NY 10036. Serotyping of *V. parahaemolyticus* should be done in reference laboratories and only to answer specific epidemiological questions.

**ANTIBIOTIC SUSCEPTIBILITY** *V. parahaemolyticus* is usually resistant to ampicillin and carbenicillin, but susceptible to colistin (Table 17). These properties are shared with *V. alginolyticus*, but *V. vulnificus* has the opposite susceptibilities (Bonner et al., 1983) (Table 15).

### *Vibrio hollisae*

*V. hollisae* is a halophilic species (Hickman et al., 1982) that is associated with diarrhea following consumption of raw seafood.

**HISTORY** *V. hollisae* was named as a new species in 1982 (Hickman et al., 1982). Two laboratories had independently studied this organism under the vernacular names “EF13” and “Enteric Group 42.” DNA hybridization studies indicated that these strains were highly related and distinct from other named *Vibrio* species, so Hickman et al. (1982) named this organism *Vibrio hollisae*.

**HABITATS** *V. hollisae* probably causes human diarrhea. Its association with diarrhea is very strong, but additional evidence is needed to further document its causal role and pathogenesis. Fifteen of the original sixteen strains were from feces, and many of the patients had diarrhea

(Hickman et al., 1982). Morris et al. (1982) described the clinical and epidemiological features of 11 diarrhea cases that were culture positive for *V. hollisae*. They also reported one isolate from blood. It was from a patient with hepatic cirrhosis, hepatic encephalopathy, bronchopneumonia, and sepsis due to the yeast *Cryptococcus*. The patient was comatose and died two days after hospital admission.

**ISOLATION** Laboratory strains of *V. hollisae* do not grow on TCBS agar (Table 4) or MacConkey agar (2 days, 36°C). Since these two media are frequently used for the isolation of *Vibrio* and Enterobacteriaceae from stools, it is possible that *V. hollisae* is often missed, even though it may be the predominant organism. *V. hollisae* grew well on sheep blood agar (36°C), so it can be detected by the use of the oxidase reagent as previously discussed.

**IDENTIFICATION** *V. hollisae* does not grow in nutrient broth without added NaCl, thus it is one of the halophilic *Vibrio* species. Tables 6 and 7 give its biochemical reactions, and only a few reactions need comment. *V. hollisae* is indole positive when tested in heart infusion broth with 1% NaCl added (Table 1), but only 38% positive in peptone water with 0.5% NaCl (the usual content). Moeller's lysine, arginine, and ornithine are all negative, which is a distinguishing characteristic (Table 6). Motility in *V. hollisae* is very slow. None of the strains were motile in semisolid medium after 48 h incubation at 36°C, but 88% were motile after 7 days. This is an unusual characteristic for the genus *Vibrio*. *V. hollisae* ferments only D-glucose, L-arabinose, D-galactose and D-mannose; this is a characteristic fermentation pattern among *Vibrio* species. Recently we confirmed a urea-positive strain of *V. hollisae*, the first one we have seen with this unusual property.

**ANTIBIOTIC SUSCEPTIBILITY** Strains of *V. hollisae* have a very characteristic antibiogram. There are very large zones around all antibiotics tested, including penicillin (Table 17).

### *Vibrio fluvialis*

*V. fluvialis* appears to cause sporadic cases of diarrhea worldwide (Lee et al., 1981; Nishibuchi and Seidler, 1983; Tacket et al., 1982), and it has been implicated in outbreaks of diarrhea in Bangladesh (Huq et al., 1980).

**HISTORY** Lee et al. (1981) gave the name *Vibrio fluvialis* to a group of halophilic vibrios that had been previously been known as "Group F vibrios" (Lee et al., 1978b) and as "Group EF6."

These organisms had been isolated from a number of environmental sources throughout the world and from humans with diarrhea. Lee et al. (1981) defined two biogroups in this species which correlated with source of isolation and certain biochemical tests. *V. fluvialis* biogroup I did not produce gas during fermentation (it was "aerogenic") and was isolated from human diarrhea as well as the environment. *V. fluvialis* biogroup II produced gas during fermentation (it was "aerogenic") and was isolated from the environment, but not from humans with diarrhea. Brenner et al. (1983b) later used DNA-DNA hybridization to show that strains of *V. fluvialis* biogroup II were related to strains of *V. fluvialis* biogroup I, but they were sufficiently different to be a new species that they named *V. furnissii*.

**HABITATS** The sources of early clinical isolates of *V. fluvialis* showed a marked association with diarrhea (Lee et al., 1981), and further studies have strengthened its causative role. One difficulty in assessing the clinical symptoms and epidemiology of *V. fluvialis* has been the presence of other possible pathogens. Most of the reports have come from geographical areas where several possible pathogens are often present in feces.

Gastroenteritis caused by *V. fluvialis* is usually described as "cholera-like." Patients typically have watery diarrhea with vomiting (97%), abdominal pain (75%), moderate to severe dehydration (67%), and often fever (35%). Usually infants, children, and young adults are affected. Frank blood is found in a small percentage of stool samples, but red or white blood cells are found in most cases (75%).

The pathogenesis of *V. fluvialis* diarrhea is not completely known. Assays for heat-labile and heat-stable enterotoxin (LT and ST) and invasiveness have generally been negative. About 20% of strains give a positive rabbit ileal loop test for enterotoxin. Recent studies have provided additional evidence for enterotoxin or for "enterotoxin-like" molecules (Lockwood et al., 1982; Nishibuchi and Seidler, 1983).

**ISOLATION** *V. fluvialis* grows on TCBS agar as 2- to 3-mm yellow colonies (Table 4). Although it requires Na<sup>+</sup> for growth, the requirement is much lower than some of the more halophilic *Vibrio* species, which makes isolation and identification easier. Nishibuchi et al. (1983) recently described a selective broth for isolating this species from water.

**IDENTIFICATION** The most striking aspect of *V. fluvialis* identification is the possible confusion with *Aeromonas*, since both species are arginine-dihydrolase positive. Many cultures sent to us

labeled “possible *V. fluvialis*” turn out to be *Aeromonas*. The converse is also true. A simple test to differentiate these two organisms is growth in nutrient broth with 0% and 1% NaCl. *V. fluvialis* is a halophilic vibrio and will not grow without the added NaCl whereas strains of *Aeromonas* grow in both media (Table 12). *V. fluvialis* is generally susceptible to antibiotics (Table 17).

### *Vibrio furnissii*

This species was formerly known as “*V. fluvialis* biovar II,” “*V. fluvialis* aerogenic,” or “*V. fluvialis* gas<sup>+</sup>.” It is now recognized as a separate species, *V. furnissii* (Brenner et al., 1983b).

**HABITATS** This species has been isolated from human clinical specimens and the environment. The vast majority of the original isolates of *V. fluvialis* (which included *V. furnissii* from patients with diarrhea) did not produce gas during fermentation. Thus, they are *V. fluvialis*.

Further investigation indicated that *V. furnissii* had been isolated from two outbreaks of acute gastroenteritis among American tourists returning from the Orient in 1969. Other pathogens or possible pathogens were also isolated (*V. parahaemolyticus*, *V. cholerae* non-O1, *V. fluvialis*, *Salmonella*, and *Plesiomonas*), so the causal role of *V. furnissii* was very doubtful. There have been no documented cases of diarrhea caused by this organism. Thus we have not included it with the other *Vibrio* species as a probable cause of diarrhea. However, microbiologists should be alert to its presence and for evidence which might show that it has a causal role in diarrhea. *V. furnissii* is apparently rare in human clinical specimens. Table 3 indicates that human stool was the most common source (16 isolates), but one isolate was from a wound. Lee et al. (1981) mentions that *V. furnissii* (listed as *V. fluvialis* biovar II) is widespread in the aquatic environment and is more common in estuaries.

**ISOLATION AND IDENTIFICATION** Table 7 indicates that *V. furnissii* resembles *V. fluvialis* very closely. The two species are so close phenotypically that only a few tests are useful in differentiating them (Tables 12 and 13). Gas production will be the key differential test in most clinical laboratories (Table 13). *V. furnissii* can also be confused with *Aeromonas* if its halophilic nature is not recognized.

### *Vibrio vulnificus*

*V. vulnificus* has been recognized as a distinct species of *Vibrio* since 1976 (Baumann and Schubert, 1984). It causes wound infections and life-threatening septicemia (Blake et al., 1979). In

the last few years, there has been intensive study of this organism (Baumann and Schubert, 1984; Blake, 1981; Colwell, 1984; Sakazaki and Balows, 1981; Tison and Kelly, 1984).

**HISTORY** Hollis et al. (1976) described a salt-requiring organism that appeared to be different from other *Vibrio* species. It was similar to *V. parahaemolyticus* and *V. alginolyticus* in many of its biochemical reactions, but the new organism fermented lactose. The vernacular name “lactose-positive *Vibrio*” was given to this organism, and in the literature it has been referred to as “L<sup>+</sup> *Vibrio*” and “Lac<sup>+</sup> *Vibrio*.” The organism was studied in several laboratories (Clark and Steigerwalt, 1977), and given the scientific name *Beneckeia vulnifica* by Reichelt et al. (1976). Classification of the organism in the genus *Beneckeia* was not widely accepted, so it was subsequently classified in the genus *Vibrio* (Farmer, 1979). Today, it is almost universally known as *V. vulnificus*.

**HABITATS** *V. vulnificus* is widely distributed in the marine environment, where some strains come in contact with humans and cause infections. This species has been associated primarily with two disease syndromes, primary septicemia and wound infection (Blake et al., 1979). Primary septicemia is a very serious infection with a fatality rate of about 50%. Most patients with primary septicemia due to *V. vulnificus* have pre-existing liver disease (Blake et al., 1979); but some were healthy individuals (Tison and Kelly, 1984). In most cases the disease begins several days after the patient has eaten raw oysters. Cultures of blood and skin lesions are usually positive for *V. vulnificus*. *V. vulnificus* also causes severe wound infections, usually after trauma and exposure to marine animals or the marine environment (Blake et al., 1979). The mortality rate is not nearly as high as in primary septicemia cases (about 7% compared to about 50%). Other infections from which *V. vulnificus* has been isolated include pneumonia in a drowning victim and endometritis which developed in a woman after exposure to seawater (Tison and Kelly, 1984).

**PATHOGENESIS** Animal studies have shown that *V. vulnificus* can cause severe local infections with gross edema leading to tissue necrosis and death. Iron availability appears to be important in pathogenesis (Tison and Kelly, 1984). A toxin produced by *V. vulnificus* has been demonstrated, although its role is still unclear.

**SOURCES** Table 3 gives the sources of our isolates. The vast majority were from blood or wound. Unusual sources included one from

urine and five from stool. *V. vulnificus* is also frequently isolated from the marine environment which is not apparent from our collection that is weighted toward human clinical specimens.

**ISOLATION** *V. vulnificus* grows well on blood agar and TCBS agar. Although most strains of *V. vulnificus* are sucrose-negative (only 3% were positive in the original report of Hollis et al., 1976), the number of sucrose-positive strains in our collection has increased markedly over the years. Thus, on TCBS agar, *V. vulnificus* can be either green (most) or yellow (some) (Table 4). This fact has not always been considered in studies looking for it in the environment. Most isolations of *V. vulnificus* in clinical laboratories will be made on blood agar, since blood and wounds are the usual sources. Commercial blood culture bottles usually support growth of *V. vulnificus*, however the Na<sup>+</sup> content is probably suboptimal, which may yield some cells with aberrant sizes and shapes. *V. vulnificus* does not survive well on media with a NaCl content of only 0.5% NaCl. Often cultures that have been shipped to us on ordinary enteric media are nonviable.

**IDENTIFICATION** *V. vulnificus* is a halophilic *Vibrio* species. It is lysine positive, and arginine negative, and is in the same group as *V. parahaemolyticus* and *V. alginolyticus* (Tables 6, 7, and 14). It is differentiated by lactose fermentation, the ONPG test, salicin and cellobiose fermentation and several other tests (Table 4). The antibiogram (disk diffusion) is also helpful in differentiating these three species (Table 14). *V. vulnificus* has either no zone or a small zone around the colistin disk, but large zones around ampicillin and carbenicillin. *V. parahaemolyticus* and *V. alginolyticus* have the opposite pattern. Although *V. vulnificus* is susceptible to most antibiotics used against Gram-negative bacteria, animal studies indicate that tetracycline may be most effective in vivo (Tison and Kelly, 1984).

### *Vibrio damsela*

*V. damsela* is a *Vibrio* species that appears to cause human wound infections. It is found in the marine environment and causes skin lesions on certain marine fish.

**HISTORY** *V. damsela* was described by Love et al. (1981). The original isolates were from skin lesions on damselfish on the California coast. Koch's postulates were fulfilled when it was documented that this organism alone caused the fish lesions. Strains of *V. damsela* were then compared to our collection of unidentified *Vibrio* strains from human clinical specimens, which revealed that *V. damsela* had occurred in human

infections (Love et al., 1981). The strains from marine fish, human clinical specimens, and the environment were studied, and *V. damsela* was proposed as a new species.

**HABITATS** *V. damsela* has been isolated from the marine environment, marine fish, and human clinical specimens. Morris et al. (1982) reviewed the case histories of six patients with wound infections whose isolates had been sent to our laboratories from 1971 to 1981. All had been acquired in coastal areas. *V. damsela* appears to cause human wound infections, but its causal role needs to be further documented. The sources of our *V. damsela* isolates are given in Table 3. All of the clinical isolates were from wounds, and 9 of the 10 were leg or foot wounds. Strains were also from marine animals, particularly marine fish. A number of fish from Dakar, Senegal, also yielded this organism. Others were from sewage, oysters, and a wound on a raccoon.

**ISOLATION** *V. damsela* grows on blood agar and other noninhibitory media, but the Na<sup>+</sup> content is less than optimum. Strains grow on TCBS agar as 2–3 mm green or yellow colonies (Table 4). Love et al. (1981) noted that the damselfish isolate grew much better on TCBS at 25°C than 36°C. There have been few details on the isolation of this organism from human clinical specimens.

**IDENTIFICATION** The complete biochemical reactions of *V. damsela* are given in Table 7. It is a typical halophilic *Vibrio* in many respects. Strains do not grow in nutrient broth without added NaCl, which indicates its requirement for Na<sup>+</sup>. *V. damsela* is arginine positive, Voges-Proskauer positive and has a characteristic fermentation pattern, positive for D-glucose, D-mannose, and usually for D-galactose and trehalose. The characteristics which differentiate it from other *Vibrio* species are given in Tables 6, 7, and 12.

**ANTIBIOTIC SUSCEPTIBILITY** *V. damsela* is generally susceptible to antibiotics, but less so than many other *Vibrio* species (Table 16).

### *Vibrio alginolyticus*

*V. alginolyticus* is very common in the marine environment (Stephen et al., 1978) and also occurs in human clinical specimens.

**HISTORY** *Vibrio parahaemolyticus* was the first of the halophilic *Vibrio* species to be studied in detail. An organism similar to, but different from, *V. parahaemolyticus* was often isolated from the marine environment, but not from patients with gastroenteritis. This organism was

originally called “biotype 2 of *V. parahaemolyticus*” (Sakazaki, 1968). However, a number of studies have shown it to be a distinct species, now universally known as *V. alginolyticus* (Sakazaki and Balows, 1981; Sakazaki et al., 1963).

**HABITAT** Many papers describe the isolation of *V. alginolyticus* from soft tissue infections (Pien et al., 1977; Rubin and Tilton, 1975). Wound infections and ear infections are usually mentioned, with eye infections mentioned much less frequently. The data show the definite association of *V. alginolyticus* with infection at these sites, however the etiological role and pathogenesis of this organism have not been thoroughly shown. However, most authors list *V. alginolyticus* as a pathogenic *Vibrio* species, particularly of wound and ear infections. Antibiotic treatment has been used in most cases and surgical debridement has been used in some.

**Table 3** gives the sources of the 74 isolates of *V. alginolyticus* in our collection. Wounds and ears are the most common sources. Interestingly, there were 12 isolates from foot or leg wounds, but only one from hand or arm wounds. *V. alginolyticus* is also widely distributed in the marine environment (Furniss et al., 1978; Sakazaki and Balows, 1981). Most clinical isolates probably come from patients exposed to the marine environment, particularly after trauma.

### Isolation and Identification

*V. alginolyticus* is a marine *Vibrio* and requires added NaCl for optimum growth. However, it grows well on many of the enteric media. It is sucrose-positive and occurs as 2–3 mm yellow colonies on TCBS agar (**Table 4**). It is easy to isolate from saltwater samples. A small volume is spread on a marine agar plate, which is then incubated at 25°C. After 18–24 of incubation, the plate is observed for colonies that swarm in a way similar to *Proteus*. A colony is restreaked onto marine agar and to TCBS agar (to prevent swarming) for purity and then identified. Many of the swarming colonies will be *V. alginolyticus* (de Boer et al., 1975a, 1975b).

**Table 7** lists the properties of this species. It is lysine-positive and arginine-negative, which puts it into the group with *V. parahaemolyticus* and *V. vulnificus* (**Table 6**). *V. alginolyticus* is usually Voges-Proskauer positive and grows in nutrient broth with 8 and 10% NaCl, which differentiates it from the other two species (**Table 4**).

**ANTIBIOTIC SUSCEPTIBILITY** *V. alginolyticus* is usually resistant to ampicillin and carbenicillin but susceptible to colistin (**Table 17**). This pattern is similar to *V. parahaemolyticus*, but *V. vulnificus* has the opposite reactions (**Table 14**).

### *Vibrio metschnikovii*

*V. metschnikovii* is a species in the genus *Vibrio* that has been frequently isolated from fresh, brackish, and marine waters, but rarely from human clinical specimens (Lee et al., 1978a).

**HISTORY** In 1884, Gamalria reported the isolation of a new organism, *Vibrio metschnikovii*, from a fowl that had died of a “cholera-like” disease (Lee et al., 1978a). Little was written about the organism until Lee et al. (1978a) began isolating similar organisms on TCBS agar from marine and freshwater environments in the United Kingdom. These strains were oxidase-negative and did not reduce nitrate to nitrite, but otherwise were quite typical halophilic *Vibrio* species. They proposed that the strains be classified as *V. metschnikovii* and wrote an amended description of this organism (Lee et al., 1978a).

**HABITATS** Lee et al. (1978a) described 40 strains of *V. metschnikovii* but none were from human clinical specimens; however, Jean-Jacques et al. (1981) reported the first clinically significant isolate of this newly redefined species. An 82-year-old woman with peritonitis and an inflamed gallbladder yielded *V. metschnikovii* from a positive blood culture. The isolate was considered clinically significant.

**Table 3** gives the sources of the isolates in our collection. An unusual finding was the two strains from urine. One was from the midstream urine of an 80-year-old woman in Canada. The other was from a routine urine culture of a 65-year-old man with chronic alcoholism, poorly controlled diabetes, and incontinence and urinary frequency. Another interesting isolate was from a wound: A 64-year-old female with peripheral vascular disease and on renal dialysis developed an ulcer around a bunion on her foot, which was treated by a wedge resection, followed by a foot amputation and finally a below-the-knee amputation. The wound yielded *V. metschnikovii* along with five other possible pathogens. The patient went fishing frequently, which may have been her source of *V. metschnikovii*. Further study is needed to determine the clinical significance of *V. metschnikovii* from sources such as urine and wound.

Nonhuman sources include rivers, sewage, cockles, shrimp, lobster, crab, and fowl (Lee et al., 1981; and our own data). These data suggest that *V. metschnikovii* is widely distributed in the environment and that humans may occasionally become colonized or possibly infected from these sources.

**ISOLATION AND IDENTIFICATION** *V. metschnikovii* grows on TCBS agar as 2–3 mm yellow colonies

(Table 4), and apparently grows well on other laboratory media. It requires Na<sup>+</sup> for growth but in much smaller amounts (5 to 15 mM) than the other Na<sup>+</sup> requiring *Vibrio* species (Baumann and Schubert, 1984). Table 7 gives the characteristics of *V. metschnikovii*. It is very easy to identify because it is oxidase negative, does not reduce nitrate to nitrite, and requires Na<sup>+</sup> for growth (Table 7). These three properties differentiate it from all the other species of Enterobacteriaceae and Vibrionaceae. *V. metschnikovii* is generally susceptible to antibiotics (Table 17).

### *Vibrio cincinnatiensis*

Little is known about this species, which was first reported by Brayton et al. (1986) from a case of bacteremia and meningitis that yielded an unusual isolate of the genus *Vibrio*. Biochemical testing and 5S ribosomal RNA sequencing led to the conclusion that this was a new species, which was named *V. cincinnatiensis*.

ISOLATION AND IDENTIFICATION *V. cincinnatiensis* grows very poorly on TCBS medium (Table 4), but isolates from extraintestinal clinical specimens should grow on one of the normal plating media such as blood agar. *V. cincinnatiensis* requires salt for growth (Table 7), and ferments myo-inositol (Table 6), which should make it easy to identify.

### *Vibrio carchariae*

Grimes et al. (1984) studied two urease-positive halophilic vibrios isolated from a brown shark (*Carcharhinus plumbeus*) that had died in captivity in a large aquarium. One of the organisms (strain 1116a) was identified as *V. damsela*, but the other (strain 1116b) could not be identified. After further phenotypic testing and DNA-DNA hybridization, Grimes et al. (1984) concluded that this was a new species and named it *V. carchariae*. *V. carchariae* has now also been found in human clinical specimens.

HABITATS *V. carchariae* has been isolated from other sharks since the original report, but it has also been shown to occur in human clinical specimens. Pavia et al. (1989) described a case in which the organism was isolated from a wound following a shark bite. An 11-year-old girl was attacked by a shark while wading in knee-deep water off the coast of South Carolina. She sustained several deep lacerations to her left calf that became infected after subsequent surgery. A culture from the infected wound yielded an unusual vibrio that was subsequently identified as *V. carchariae*.

ISOLATION AND IDENTIFICATION *V. carchariae* appears to grow on plating media such as chocolate agar and MacConkey agar (Pavia et al., 1989), and grows well on TCBS agar as yellow colonies (Table 4). It is a halophilic vibrio and is similar to *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus* in its biochemical properties, but can be differentiated (Table 14). Strains are resistant to ampicillin, carbenicillin, and colistin (Table 17).

## ***Vibrio* Species That Do Not Occur in Human Clinical Specimens**

In addition to the 12 *Vibrio* species discussed above that occur in clinical specimens, there are over 20 marine species that apparently do not. Theoretically, these marine vibrios could occur in feces following ingestion of environmental water or uncooked seafood, and we have some evidence that this occurs. Many of the species have few literature citations (see Table 1 in Chapter 156), and this group has not been studied as thoroughly as the human pathogens. Table 15 gives the phenotypic properties of these species based on the methods used in our laboratory. Some of the biochemical test results at 25°C in media with added marine cations differ from those originally published. This may reflect the different methods used since these types of differences are not unusual in “between-laboratory” comparisons. These marine species are briefly described in the following sections.

### *Vibrio anguillarum*

*V. anguillarum* is a marine vibrio that causes disease in marine fish and other marine animals (Baumann et al., 1978; Sakazaki and Balows, 1981; Smith, 1961). The most important is a septicemic disease in marine fish characterized by a deep necrotizing myositis and subdermal hemorrhages. This disease is particularly important when it occurs at fish farms because the economic losses are world-wide and considerable. The main predisposing factor seems to be a rise in water temperature to 15°C or more (Sakazaki and Balows, 1981). *V. anguillarum* may also cause a necrotic disease of bivalve mollusks’ larvae and juveniles (Tubiash et al., 1970).

*V. anguillarum* grows on many bacteriological media if the NaCl content is 1 to 2%, but most strains do not grow well on TCBS agar (Sakazaki and Balows, 1981). BTB teepol agar, originally described for the isolation of *V. parahaemolyticus*, is a good isolation medium for specimens obtained from diseased fish.

## BTB-Teepol Agar (Akiyama et al., 1963)

Beef extract	5 g
Peptone	10 g
Sucrose	10 g
Sodium chloride	20 g
Teepol (Shell Chemical Co.)*	2 ml
Bromthymol blue	80 mg
agar	15 g
water	1 liter

Adjust the pH to 7.8. Dissolve the ingredients by gentle heating. Autoclave at 121°C for 15 min, and pour into plates. *V. anguillarum* grows as moderate-sized yellow colonies (Sakazaki and Balows, 1981), but other vibrios also grow, so complete identification will be required for the colonies picked.

\*Sakazaki and Balows (1981) indicate that Turgitol 7 may be substituted.

Identification of *V. anguillarum* may be difficult because strains vary considerably in their phenotypic properties (Table 15). Strain from fish disease with this biochemical pattern are likely to be *V. anguillarum*.

*Vibrio ordalii*

*V. ordalii* is a halophilic *Vibrio* species that is very closely related to *Vibrio anguillarum* and was named by Schiewe et al. (1981). Previously, other investigators had studied vibriosis of marine fish and isolated a halophilic vibrio similar to, but less biochemically active (Table 15) than *V. anguillarum*. This organism was referred to as “*Vibrio* sp. 1669 group,” “*Vibrio* sp. RT group” *Beneckea anguillara* biotype 1, or *Vibrio anguillarum* biotype 2. However, the DNA-DNA hybridization studies of Schiewe et al. (1981) indicated that, although it was closely related to *V. anguillarum*, it was a separate species. *V. ordalii* has been isolated from diseased salmonid fish in Washington, Oregon, British Columbia, and Japan reference is not an exact match (Schiewe et al., 1981), and from ayu (*Plecoglossus altivelis*) and rockfish fingerlings (*Sebastes schlegeli*) in Japan (Muroga et al., 1986). The GC content of the DNA is 43–44 mol%.

*Vibrio aestuarianus*

*V. aestuarianus* is a halophilic *Vibrio* species that was first described by Tison and Seidler (1983) who isolated it from water and shellfish off the coast of Oregon. Strains are arginine-positive, Voges-Proskauer-negative, sucrose-positive, and lactose-positive, and do not grow in media with 0 or 5% NaCl. The GC content of the DNA is 43–44 mol%.

*Vibrio campbellii*

*V. campbellii* is a halophilic *Vibrio* species that was first described by Baumann et al. (1971a)

who isolated 60 strains from ocean water off the coast of Hawaii. They noted considerable variation in the phenotypic properties of this large group, but included all of them in *V. campbellii*. The GC content of the DNA is 46–48 mol%, and this species is about 65% related by DNA-DNA hybridization to *Vibrio harveyi* (Reichelt et al., 1976). Grimes et al. (1986) isolated 20 strains from seawater during a voyage from Barbados to Puerto Rico to Bermuda, and concluded that *V. campbellii* is also found in the open waters of the Atlantic ocean.

*Vibrio costicola*

*V. costicola* was first described by Smith (1938). It is a unique *Vibrio* species because of its large requirement for and tolerance to NaCl. Strains usually will not grow in laboratory media unless the NaCl content is 200–250 mM (1.17 to 1.46% NaCl), which defines *V. costicola* as a moderate halophile. Strains have been isolated from environments with a very high salt content, such as meat-curing brines and solar salterns (Garcia et al., 1987). *V. costicola* has been a popular organism in physiological studies, particularly those comparing different halophilic bacteria or comparing halophilic with nonhalophilic species. The GC content of the DNA is 50 mol%.

*Vibrio diazotrophicus*

*V. diazotrophicus* is a halophilic *Vibrio* species that was first described by Guerinot and Patriquin (1981), who isolated nitrogen-fixing vibrios from sea urchins (*Strongylocentrotus droebachiensis*) in Nova Scotia. This group of three strains plus 10 additional ones was named *Vibrio diazotrophicus* by Guerinot et al. (1982) because it was not highly related to other vibrios by DNA-DNA hybridization. An additional nitrogen-fixing species isolated from a sea urchin (*Triploneustes ventricosus*) in Barbados was only 14% related by DNA-DNA hybridization, so it was excluded from the species. The ability to fix molecular nitrogen seems to be a unique property for this species of *Vibrio*. Other strains have been isolated from ditch water, sediment, and reeds at Chetney Marsh, England; the Humbler River (England), and Chesapeake Bay water. The GC content of the DNA is 46–47 mol%.

*Vibrio fischeri*

*V. fischeri* is a halophilic *Vibrio* species that is also bioluminescent. It was first described as *Photobacterium fischerii* by Beijerinck (1989) and later classified in the genus *Vibrio* by Lehmann and Neumann (1896). It was named for Bernhard Fisher who was one of the first to sys-



tematically study bioluminescent bacteria. Strains grow at 30°C but many can not grow at 37°C. Many strains produce yellow pigment. *V. fischeri* has been isolated from many geographical locations and from coastal and open ocean seawater, surfaces and feces of fish and squids, luminous organs (Baumann and Baumann, 1981), and seafood (Furniss et al., 1978). The GC content of the DNA is 39–41 mol%.

#### *Vibrio gazogenes*

*V. gazogenes* is a halophilic *Vibrio* species that produces a characteristic dry red colony on marine agar that makes it easy to isolate and identify. It was first described as *Beneckea gazogenes* by Harwood (1978), but was reclassified in the genus *Vibrio* when the genus *Beneckea* was abolished. Strains produce gas during fermentation but do not reduce nitrate to nitrite, which also proves very useful in identification. *V. gazogenes* has been isolated from salt marshes in Massachusetts (Harwood, 1978), North Carolina, and South Carolina (Farmer et al., 1988) and from a hypersaline basin at Laguna Figueroa, Baja California de Norte, Mexico (Giovannoni and Margulis, 1981). The GC content of the DNA is 47 mol%.

#### *Vibrio harveyi*

*V. harveyi* is a halophilic *Vibrio* species that was first described as *Achromobacter harveyi* by Johnson and Shunk (1936), who named it for E. N. Harvey, a pioneer in the study of bioluminescence. It has been classified in *Lucibacterium* and *Beneckea*, but is now included in *Vibrio*. Many strains are bioluminescent, but nonluminescent strains occur also and can be difficult to distinguish from other vibrios (Furniss et al., 1978), particularly *V. vulnificus* (Yang et al., 1983). *V. harveyi* has been isolated from many geographical locations and from coastal and open ocean seawater, and surfaces and feces of fish and squids (Baumann and Baumann, 1981). Many papers have been published on its physiology and metabolism, and it is often used in bioluminescence studies. The GC content of the DNA is 46–48 mol%.

#### *Vibrio logei*

*V. logei* is a halophilic *Vibrio* species that was first described as *Photobacterium logei* by reference is not an exact match Bang et al. (1978) who studied a group of strains that had been isolated from a variety of marine sources. Strains of *V. logei* are bioluminescent, yellow-pigmented, and grow at 4 and 15°C, but not at 30°C. Many of the isolates of *V. logei* were obtained in the Arctic, and

sources include exoskeleton lesions of tanner crabs, fish intestinal contents, scallops, and marine sediments (Baumann and Baumann, 1981). The GC content of the DNA is 40–42 mol%.

#### *Vibrio marinus*

*V. marinus* is a halophilic *Vibrio* species that was first described as *Spirillum marinum* by Russell (1891), who isolated it from samples taken in the Gulf of Naples (Ford, 1927; Colwell and Morita, 1964). Colwell and Morita (1964) described 16 strains of *V. marinus* that were isolated from Puget Sound on the Pacific coast of the United States. However, only one strain (ATCC 15381, from seawater) is still available, and there has been limited work on this species. *V. marinus* is a psychrophilic marine vibrio and grows at 4 and 20°C, but not at 30°C (Baumann et al., 1984). The GC content of the DNA is 42 mol%.

#### *Vibrio mediterranei*

*V. mediterranei* is a halophilic *Vibrio* species that was first described by Pujalte and Garay (1986), who isolated it from plankton, sediments, and seawater in two coastal areas south of Valencia, Spain (Pujalte et al., 1983). There are no outstanding characteristics for *V. mediterranei*, so complete biochemical testing is required to distinguish it from other marine vibrios (Table 4). The GC content of the DNA is 42–43 mol%.

#### *Vibrio natriegens*

*V. natriegens* is a halophilic *Vibrio* species that was first described as *Pseudomonas natriegens* by Payne et al. (1961), who isolated it from salt marsh mud on Sapelo Island off the coast of Georgia. *V. natriegens* uses a wider variety of carbon sources than other marine vibrios (Baumann and Baumann, 1984), which make it easy to isolate and identify. It also has the shortest generation time (9.8 min) of any bacterium (Eagon, 1962), which makes it a popular species to use in teaching exercises and physiological studies. *V. natriegens* has been isolated from coastal seawater in several locations (Baumann and Baumann, 1981; Furniss et al., 1978). The GC content of the DNA is 46–47 mol%.

#### *Vibrio nereis*

*V. nereis* is a halophilic *Vibrio* species that was first described as *Beneckea nereida* by Baumann et al. (1971a), who isolated it from seawater off the coast of Oahu, Hawaii. *V. nereis* accumulates PHB is positive for arginine dihydrolase, and does not grow on TCBS agar (Furniss et al.,

1978), but is otherwise similar to other marine vibrios. Kusuda et al. (1986) isolated *V. nereis* from an outbreak of fatal infections of sea bream (*Acanthopagrus schlegeli*) at hatcheries in western Japan, but did not think this organism was actually infecting the fish. The GC content of the DNA is 46–47 mol%.

### *Vibrio nigripulchritudo*

*V. nigripulchritudo* is a halophilic *Vibrio* species that was first described as *Beneckeia nigrapulchrituda* by Baumann et al. (1971b), who isolated it from seawater off the coast of Oahu, Hawaii. Colonies of *V. nigripulchritudo* produce a distinct blue-black pigment which simplifies its isolation and identification. The GC content of the DNA is 46–47 mol%.

### *Vibrio orientalis*

*V. orientalis* is a halophilic *Vibrio* species that was first described by Yang et al. (1983), who isolated it from seawater and shrimp off the coast of China. No other isolates have been reported in the literature. All the original strains of *V. orientalis* were reported to be bioluminescent, but in our hands strains (which had been freeze-dried) have been weak or negative. Strains of *V. orientalis* grow at 4, 30, and 35°C, accumulate PHB, and are arginine positive. However, it can easily be confused with other marine vibrios (Table 15). The GC content of the DNA is 45–46 mol%.

### *Vibrio pelagius*

*V. pelagius* is a halophilic *Vibrio* species that was first described as *Beneckeia pelagia* by Baumann et al. (1971a) who isolated it from seawater off the coast of Oahu, Hawaii. Few additional isolations have been reported in the literature; however, Furniss et al. (1978) indicate that *V. pelagius* is one of the most common vibrios isolated around the British coasts. This species is subdivided into two groups based on DNA-DNA hybridization and phenotypic properties, and can easily be confused with other marine vibrios. The GC content of the DNA is 45–47 mol%.

### *Vibrio proteolyticus*

*V. proteolyticus* is a halophilic *Vibrio* species that was first described as *Aeromonas proteolytica* by Merkel et al. (1964), who isolated it from *Limnoria tripunctata* (a small, wood-boring isopod crustacean) collected from wood pilings at Fort Jackson Marine Biological Laboratory, Charleston, South Carolina. This is the only strain described in the literature. This strain of *V. proteolyticus* swarms on complex marine media, is

highly proteolytic and Voges-Proskauer positive, but can easily be confused with other marine vibrios (Table 15). The GC content of the DNA is 50.5 mol%.

### *Vibrio salmonicida*

*V. salmonicida* is a halophilic *Vibrio* species that causes a serious disease (“Hitra disease” or “cold-water vibriosis”) of Atlantic salmon (*Salmo salar*) and rainbow trout (*Salmo gairdneri*) in Norwegian salmonid farms (Egidius et al., 1981). This organism had a number of vernacular names until it was named *V. salmonicida* by reference is not an exact match Egidius et al. (1986), who showed that it was not closely related to other *Vibrio* species by DNA-DNA hybridization (Wiik and Egidius, 1986). Strain of *V. salmonicida* grow slowly on laboratory media and have a very low growth temperature range of 1–22°C. Its psychrophilic nature, very slow growth rate, and narrow ecological niche are helpful in isolation and identification. The GC content of the DNA is 42 mol%.

### *Vibrio splendidus*

*V. splendidus* is a halophilic *Vibrio* species that was first described as *Photobacter splendidum* by Beijerinck (1900). This species is subdivided into two groups by DNA-DNA hybridization and phenotypic properties, and can easily be confused with other marine vibrios (Table 15). *V. splendidus* biogroup 1 strains are bioluminescent and positive for arginine dihydrolase, but strains of biogroup 2 are negative for both tests. Strains of *V. splendidus* have been isolated from seawater and fish on the east coast of North America and Denmark (Baumann and Baumann, 1981). The GC content of the DNA is 45–46 mol%.

### *Vibrio tubiashii*

*V. tubiashii* is a halophilic *Vibrio* species that was first isolated by Tubiash et al. (1965), who was studying bacillary necrotic disease of larval and juvenile bivalve mollusks. These authors identified the causative bacterium as *Vibrio anguillarum* and deposited three strains (ATCC 19105, 19106, and 19109) in the American Type Culture Collection. However, Hada et al. (1984) studied these three strains and showed that they belonged to a new species they named *V. tubiashii*, in honor of H. S. Tubiash. This species does not have any outstanding phenotypic properties to distinguish it from other marine vibrios, but seems to be isolated only from diseased marine mollusks. It is pathogenic for larvae of three oyster species (Hada et al., 1984), *Crassostrea virginica*, *Crassostrea gigas*, and *Ostrea edulis* (Lodeiros et al.,

1987); larvae of the clam *Mercenaria mercenaria* (Hada et al., 1984); and for the lesser octopus *Eledone cirrhosa* (Bullock et al., 1987). The GC content of the DNA is 43–45 mol%.

## The Genus *Photobacterium*

The genus name *Photobacterium* was first used by Beijerinck (1889), and for much of its history the genus has been based on a single characteristic, bioluminescence, the ability to produce light. As time passed, nonfermentative and Gram-positive bioluminescent species were removed from the genus, but only in the decade has it had a more narrow definition based on evolutionary relationships (Baumann and Baumann, 1981, 1984). Today, most authorities recognize only three species in the genus: *P. phosphoreum*, *P. leiognathi*, and *P. angustum*. Because other bioluminescent bacteria have been included in the genus *Photobacterium* for so much of its history, it is very difficult to identify the true species. Table 18 lists the differential and other properties of the three *Photobacterium* species.

### History, Nomenclature, and Classification

A general discussion of bacterial bioluminescence is given in *The Luminous Bacteria* in the second edition. Bacterial bioluminescence dates back to ancient times, but its bacterial nature has been known for only a hundred years (Sieburth, 1979). Aristotle recorded that dead fish could sometimes produce light, and Robert Boyle noted that air was required for light production. However, it was not until the late 1880s that studies were done on bioluminescent bacteria (ZoBell, 1946).

Luminescent bacteria were isolated from a variety of marine samples by several early bacteriologists, and Pflüger (1875) may have been the first of these (ZoBell, 1946). He isolated luminescent bacteria from fish, but did not name any species. Bernard Fischer, for whom *Photobacterium fischeri* (now *Vibrio fischeri*) is named, served as a ship's physician on the voyage of the S. M. S. Molthe from the Baltic Sea to the West Indies and made a number of discoveries in marine microbiology (ZoBell, 1946). He isolated a bioluminescent organism from waters in the West Indies and named it *Photobacterium indicum* (Fischer, 1887).

Over the next hundred years, a large number of bioluminescent bacteria were isolated from different sources, mostly marine. Many of these organisms were given scientific names and classified in the genus *Photobacterium*. *Index Bergeyana* (Buchanan et al., 1966, p. 803–806)

lists over 40 different *Photobacterium* species, and the *Supplement to Index Bergeyana* (Gibbons et al., 1981, p. 201–202) lists an additional 10 species. The original descriptions of most of these organisms are sketchy, but some are probably synonyms of the current *Photobacterium* species or of bioluminescent *Vibrio* species (Baumann and Baumann, 1981).

The genus *Photobacterium* as it is currently defined is based on the studies of Reichelt et al. (1976), who used DNA-DNA hybridization to compare several species of *Photobacterium* and *Vibrio* (Baumann and Baumann, 1981). They examined *P. phosphoreum* strain 404 and found that it was 84% related (at a hybridization temperature of 63°C) to the type strain of this species (strain 439 = ATCC 11040) and 92 to 99% related to three other strains of this species (Fig. 16). *P. phosphoreum* strain 404 was 22–36% related to five strains of *P. angustum*, and 24–36% related to five strains of *P. leiognathi*. However, these latter two *Photobacterium* species were more closely related to *P. phosphoreum* than was *V. harveyi* (11–13%) (called *Beneckea harveyi* in the paper) *V. fischeri* (5–12%), *V. cholerae* (0%), and six other *Vibrio* species (0–5%). The close relatedness of the three *Photobacterium* species is the basis of the genus as it is now defined; however, *P. angustum* and *P. leiognathi* are much more closely related to each other than they are to *P. phosphoreum* (Fig. 16).

In their chapter for the first edition of this handbook, Baumann and Bauman (1981) included *Vibrio fischeri* and *V. logei* (Bang et al., 1978a) in the genus *Photobacterium*, but in the Addendum they reclassified both of these species in the genus *Vibrio*. This latter classification was in better agreement with the morphological, phenotypic, and molecular data.

### Description of the Genus *Photobacterium*

The following description now applies to the genus: small, plump, rod-shaped bacteria, Gram-negative, motile with one to three unsheathed polar flagella (Fig. 14, part 5; Fig. 15, parts 11 to 15), usually bioluminescent (two of the three species), accumulate large amounts of PHB when grown in a basal medium with glucose as the sole source of carbon (Fig. 15, part 13), do not form PHB when grown in rich media containing peptone, grow in the presence and absence of oxygen, are oxidase-positive (2 of 3 species) and catalase-positive, reduce nitrate to nitrite, grow with glucose as the sole source of carbon and  $\text{NH}_4^+$  as the sole source of nitrogen (some strains require amino acids for growth, see Table 18), require  $\text{Na}^+$  for growth, metabolism is fermentative rather than oxidative, and one species produces gas during fermentation.

Table 18. Differential and other properties of the three species of *Photobacterium*.<sup>a</sup>

Property or test	<i>P. phosphorium</i>	<i>P. leiognathi</i>	<i>P. angustum</i>
Association with fish family			
Leioagnathidae	+	–	–
Apogonidae	+	–	–
Macrouridae	–	+	–
Merluccidae	–	+	–
Opisthoproctidae	–	+	–
Trachichthyidae	–	+	–
Moridae	–	+	–
Growth at:			
4°C	96	0	(+)
20°C	+	+	+
25°C	99	100	
30°C	85	100	+
35°C	0	96	+
40°C	0	0	–
Cell morphology			
Straight rods	100	96	
Curved rods	0	4	
Poly-β-hydroxybutyrate accumulation	100	100	
Flagellation pattern			
Number of unsheathed polar flagella	1–3	1–3	
Sheathed polar or peritrichous flagella	–	–	
GC content (from Reichelt and Baumann, 1973)	41.2–41.8	42.8–43.3	
GC content (from Baumann and Baumann, 1984)	41–42	42–44	40–42
Require Na <sup>+</sup> (100 mM) for optimum growth	+	+	+
Oxidase (R1)	7	64	
Bioluminescent	+	+	–
Luciferase kinetics (from Hastings and Nealson, 1981)	Fast	Fast	–
Nitrate reduced to nitrite	93	96	V
Gas production during fermentation of D-glucose	89	7	–
Voges-Proskauer	97	93	
Growth on glucose as the sole source of carbon	58		
Nutritional requirements			
L-Methionine alone	24	0	–
L-Histidine alone	1	0	
L-Arginine alone	0	4	
L-Methionine plus one or two other amino acids	15	0	
Amino acid mixture	5	0	
Vitamins (yeast extract)	1	0	
Extracellular enzymes			
Gelatinase	0	0	(+)
Lipase	0	82	V
Chitinase	97	96	–
Alginase	0	0	–
Amylase	0	0	–
Growth on carbon sources:			
D-Xylose	–	–	+
Maltose	99	0	V
Acetate	0	86	+
DL-Glycerate	+	V	–
DL-Lactate	18	100	
Pyruvate	0	96	+
L-Proline	4	96	–
D-Glucuronate	49	0	

<sup>a</sup>The numbers give the percentage positive for *P. phosphorium* (74 strains studied) and for *P. leiognathi* (28 strains). Adapted from Reichelt and Baumann (1976).

## Habitats

*Photobacterium* and other bioluminescent bacteria are widely distributed in the marine environ-

ment (Baumann and Baumann, 1981, 1984), and one must be careful to determine which reports are using the terms *Photobacterium* and bioluminescent bacteria interchangeably. Biolumines-

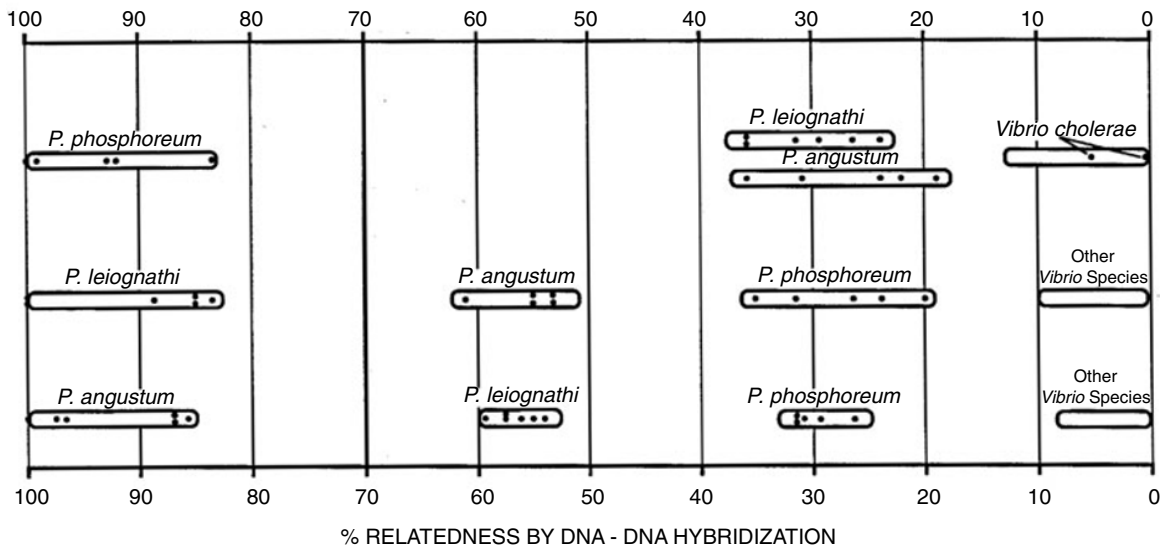


Fig. 16. Relatedness of the three species of *Photobacterium* by DNA-DNA hybridization; note that *P. angustum* and *P. leiognathi* are more closely related to each other than they are to the type species for the genus, *P. phosphoreum*.

cent bacteria are very common in marine water samples, and are presumably shed into the water from more protected ecological niches (Hastings and Nealson, 1977, 1981; see also *The Luminous Bacteria* in the second edition).

In addition to their common occurrence on fish and other marine animals, luminescent bacteria are found in the light organs (Fig. 17) of many marine animals (Bassot, 1975; Boisvert et al., 1967; Harvey, 1940; Hastings and Nealson, 1977, 1981). Two of the species of *Photobacterium* inhabit the light organs of marine fish. *P. phosphoreum* inhabits the light organs of five fish families (Table 18) that are usually found in deep cold waters. This ecological niche agrees with the fact that this species grows at 4°C, but poorly at 25°C and not at 30 or 37°C. *P. phosphoreum* is clearly a psychrophilic deep-water species. Reichelt and Baumann (1973) found it at depths of 50 to 500 off the coast of Hawaii. *P. leiognathi* inhabits the light organs of two fish families (Table 18) that are usually found in warm, shallow, tropical water (Hastings and Nealson, 1981). This species grows at higher temperatures, (Table 18) which agrees with this warmer habitat. In their review of *Photobacterium*, Baumann and Baumann (1984) mention that the only isolates of *P. angustum* have been from the open ocean off Hawaii. However, both *P. angustum* and *V. logei* have been reported recently from a marine fish-rearing unit (Austin, 1983).

Hastings and Nealson (1981) point out that light emission by bioluminescent bacteria may not directly benefit the bacterium, but may be important to its partner in a mutualistic symbiotic relationship. The symbiotic bioluminescent bacteria provide an excellent example of this rela-

tionship where the partner is usually a marine fish. The bacteria are localized in a light organ where they emit light. The review of the symbiotic bioluminescent bacteria by Hastings and Nealson (1981) is highly recommended (see also *The Luminous Bacteria* in the second edition).

Bioluminescent bacteria are very numerous in the intestinal tract of marine fish and often comprise close to 100% of the aerobic heterotrophic flora. They are also found on the gills, and in lower numbers on the skin (Baumann and Baumann, 1981, 1984).

### Isolation

Strains of *Photobacterium* and bioluminescent *Vibrio* species can easily be isolated from seawater and marine animals (Baumann and Baumann, 1981; Hastings and Nealson, 1981). The best sources of *P. phosphoreum* and *P. leiognathi* are the luminous organs of marine fish (Table 18), where they are present in high numbers and are often in pure culture.

Seawater or similar marine media are usually required for growth and optimum light production. Luminescence and respiration are reduced in medium when seawater is diluted by more than 50% with fresh water. Isolation of luminescent vibrios has been discussed previously; there are no specific media to select for *Photobacterium* species at the expense of other vibrios.

### Identification

The species of *Photobacterium* are phenotypically very similar to the marine species of *Vibrio* (Table 15; also see Table 4 in *The Family*

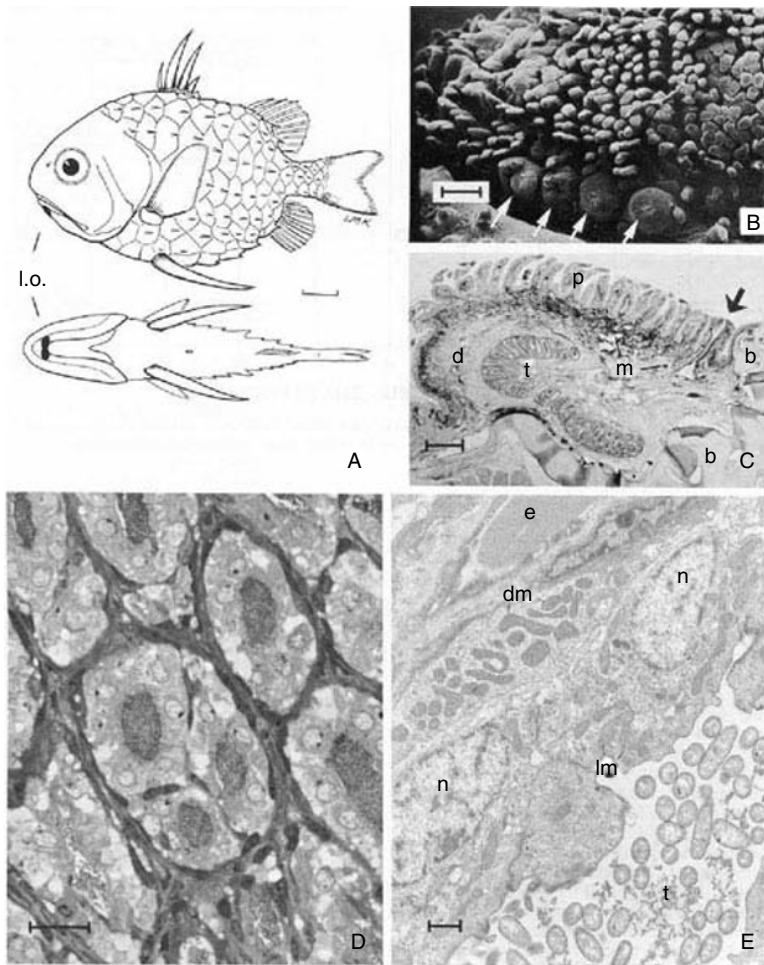


Fig. 17. *Monocentris japonicus*. (A) Line drawing of fish and ventral view of lower jaw, showing location of light organs (l.o.). Bar = 1.0 cm. (B) Scanning electron micrograph of the dorsal surface of the light organ. Numerous dermal papillae can be seen. The emissary ducts from the light organ emerge at the tips of the four large dermal papillae (arrows). Bar = 0.2 mm. (C) Light micrograph of a sagittal section of the lower jaw. m, melanocytes; t, tubules with bacteria; b, mandibular bone; d, dermal layer; p, dermal papillae. Arrow points to emissary duct. Bar = 50  $\mu$ m. (D) Light micrograph showing the light organ tubules filled with bacteria. Tubules are lined with a single layer of cuboidal epithelial cells that display loose nuclear chromatin and prominent nucleoli supported by connective tissue cells. Blood capillaries are sparse and not readily visible. Bar = 15  $\mu$ m. (E) Electron micrograph showing the major features of tubule epithelium. Epithelial cells that make up the lining of the tubules have light-staining mitochondria (lm) with fine cristae. Epithelial cells that are further away from the tubule lumen next to the blood capillaries have dark-staining mitochondria (dm) with thick cristae. t, tubule containing luminous bacteria; e, erythrocyte visible in capillary; n, nucleus of tubule epithelium cells. Bar = 1  $\mu$ m.

Vibrionaceae in this Volume). For this reason it can be difficult to differentiate them (Baumann and Baumann, 1981; Hastings and Nealson, 1981). Samples from the light organs of certain marine fish (Table 18) are likely to yield either *P. phosphoreum* or *P. leiognathi*. Isolates from seawater or decaying organic matter are more difficult to identify to species because they could be one of the *Photobacterium* species or one of the other bioluminescent vibrios. Table 15 gives the biochemical reactions of the three species of *Photobacterium* and the species of *Vibrio* that can be confused with them. Several of the tests require comment.

**Biochemical and Other Tests (Baumann and Baumann, 1981; Hastings and Nealson, 1981)**

**OXIDASE** The results for this test are variable for the three species of *Photobacterium*, in contrast to most strains of *Vibrio*, *Aeromonas*, and *Plesiomonas*, which are strongly oxidase-positive. Strains of *Photobacterium* apparently contain

low levels of cytochrome *c*, the compound that is responsible for a positive oxidase reaction. Strains that are oxidase negative should be retested after treating the cells with toluene (Baumann and Baumann, 1981). Toluene treatment presumably causes cell lysis and liberation of internal proteins. Many oxidase-negative strains of *Photobacterium* are positive with this modification.

**FLAGELLA STAINS** Reichelt et al. (1976) showed that tufts of polar flagella often tended to fold backwards, giving the appearance of peritrichous flagella, especially in Leifson (1960) flagella stains.

**ARGININE DIHYDROLASE-DECARBOXYLASE** Strains of *Photobacterium* sometimes produce an alkaline reaction in arginine decarboxylase test media, such as Moeller's medium used in clinical microbiology laboratories, even though they lack the enzyme arginine dihydrolase (Baumann and Baumann, 1984). *P. phosphoreum* apparently decarboxylates and deaminates arginine.

**NUTRITIONAL REQUIREMENTS** These tests may be helpful for identifying *Photobacterium* strains, particularly those that have been maintained in the laboratory. Most freshly isolated strains of *Photobacterium* and other luminescent bacteria do not require vitamins or amino acids (Ruby and Nealson, 1978), but strains that have been maintained in the laboratory may have a requirement (Baumann and Baumann, 1981) (Table 18). Frequent transfers on complex media probably lead to these nutritional defects.

**DARK (NONBIOLUMINESCENT) MUTANTS OF BIOLUMINESCENT STRAINS** Strains may become nonbioluminescent when they are maintained in the laboratory or in culture collections in contrast to the original strains that are brightly luminescent.

**CARBON SOURCES** In contrast to most *Vibrio* species that are nutritionally very versatile, the species of *Photobacterium* can use only a few compounds as sources of carbon and energy (Baumann and Baumann, 1981, 1984).

**TURNOVER KINETICS OF THE ENZYME LUCIFERASE** Hastings and Mitchell (1971) showed that the enzyme luciferase differs in its "turnover kinetics" depending on the bioluminescent species from which it is isolated. The luciferase from *V. harveyi* has slow turnover kinetics, in contrast to the fast turnover kinetics of *V. fischeri* and *P. leiognathi*. At one time this difference was used as a characteristic for differentiating the genus *Photobacterium* from the bioluminescent species of *Vibrio* (Hastings and Nealson, 1981).

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## The Genera *Aeromonas* and *Plesiomonas*

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Traditionally the genera *Aeromonas* and *Plesiomonas* have been considered together. Three international workshops (International Workshop, 1986, 1988, 1990) have considered many different aspects of these two genera. In addition, the two genera are usually included in the same chapter or review (von Graevenitz, 1985). Only recently, however, has the relationship of the two genera been studied with methods that measure evolutionary distance. Although *Aeromonas* and *Plesiomonas* are not closely related, we have taken the traditional approach and considered them together in this chapter. However, we have separated most of our discussion because their differences seem more important than their similarities.

### The Genus *Aeromonas*

The genus *Aeromonas* has been known to microbiologists for many years. Most of the early literature was about diseases of frogs, fish, and other animals. *Aeromonas* is now recognized as an important opportunistic pathogen of humans; however, there is a controversy on the role of *Aeromonas* as a cause of human diarrhea. Through 1989, there were 1,436 literature citations for *Aeromonas* in the *Medline* computer data base and 2,589 citations in BIOSIS, so the reader interested in a particular aspect can use a computer literature search as a starting point.

### History, Nomenclature, and Classification of *Aeromonas*

The nomenclature and classification of the genus *Aeromonas* has been in a state of flux since these organisms were first described in the early 1890s and it is often difficult to know which *Aeromonas* species (in today's usage) a particular article in the literature deals with. We have divided the history of *Aeromonas* into three different time periods. In the period 1890–1936, many different names were proposed for the *Aeromonas* strains isolated from a wide variety of sources and dis-

eases. In the period 1936–1979, the genus *Aeromonas* as we know it today was established, and many of the described species were studied and compared using techniques that measure phenotypic similarities and differences, but not evolutionary distance. Since 1979, techniques that measure evolutionary distance have been used to compare the species and to determine their relationship to other bacteria. These techniques have yielded some surprising results that form the basis of a more logical classification for the genus *Aeromonas*.

#### *Aeromonas* from 1890 to 1936

The first description of an organism that is now known to be an authentic *Aeromonas* species perhaps was that of *Bacillus hydrophilus fuscus* by Sanarelli (1891). Sanarelli (1891) was studying immunity to the anthrax bacillus in frogs, but many of his animals developed a septicemia that was not caused by the anthrax bacillus. Sanarelli isolated the causative bacterium and named it *B. hydrophilus fuscus*.

Other *Aeromonas*-like organisms were also described during this period by workers studying septicemic diseases of frogs (Ernst, 1890; Sanarelli, 1891). Other strains were soon isolated from water (Beijerinck, 1900; Zimmerman, 1890), milk (Hammer, 1917), and diseased trout (Emmerich and Weibel, 1894). The methods used to characterize and identify species during this period were very primitive so it is very difficult to determine if different authors are writing about the same organism. Even in the early literature it was clear that two different groups of *Aeromonas* were being studied. Most of the strains were motile, did not produce a brown diffusible pigment, and grew well at 37°C. Today these strains comprise the *Aeromonas hydrophila* group. Strains of a different group were isolated from diseased fish.

**PATHOGENIC *AEROMONAS* STRAINS ISOLATED FROM FISH** An organism that Emmerich and Weibel (1894) called “*Bacillus der Forellenseuche*” was isolated from diseased trout in 1894. Since then, similar bacteria have been isolated from furunculosis lesions in fish. These bacteria were referred to by Lehmann and Neumann (1896)

as *Bacterium salmonicida*. This organism was described as being a Gram-negative, nonmotile bacillus that liquefied gelatin and had an optimum growth temperature of 10–15°C. It produced a brown pigment in media containing tyrosine and did not grow at 37°C. Today this organism is known as *Aeromonas salmonicida*, an important fish pathogen. Most classifications of *Aeromonas* have treated *A. salmonicida* as a separated named species, which has been the one stable factor in the many classifications proposed for *Aeromonas*.

**EARLY CLASSIFICATIONS OF *AEROMONAS*** Prior to 1920, organisms that currently belong to the genus *Aeromonas* were classified in the genera *Bacillus* (Chester, 1901; Sanarelli, 1891; Zimmerman, 1890), *Bacterium* (Hammer, 1917), and *Aerobacter* (Beijerinck, 1900). After 1920, the taxonomy became more confusing. Species were classified in the genera *Proteus* (Bergey et al.,

1923; 1925, 1934; Miles and Halnan, 1937; Pribham, 1933), *Pseudomonas* (Breed et al., 1948; Crawford, 1954; Scherago, 1936, 1937), *Escherichia* (Bergey et al., 1923), *Achromobacter* (Bergey et al., 1925), *Flavobacterium* (Bergey et al., 1934), *Bacterium* (Weldin and Levine, 1923), *Necromonas* (Smith, 1963), *Vibrio*, and *Aeromonas*. Many of the species named are actually synonyms of other species (Table 1). During the early 1920s, other species were described but there was little agreement on classification. In the 1930s, there were other changes in the nomenclature and classification of aeromonads (Bergey et al., 1934; Pribham, 1933a, 1933b), but the main contribution was the first use of the genus name *Aeromonas* (Kluyver and van Niel, 1936).

#### *Aeromonas* from 1936 to 1979

The genus name *Aeromonas* was first used by Kluyver and van Niel (1936) in their “natural

Table 1. Synonyms of the species of the genus *Aeromonas*.

Species	Synonym	Reference <sup>a</sup>
<i>A. hydrophila</i>	<i>Bacillus hydrophilus fuscus</i>	Sanarelli, 1891
	<i>B. ranicida</i> Ernst <sup>b</sup>	Lehmann and Neumann, 1896
	<i>Aerobacter liquefaciens</i>	Beijerinck, 1900
	<i>B. hydrophilus</i> Sanarelli	Chester, 1901
	<i>B. ichthyosmius</i>	Hammer, 1917
	<i>Proteus hydrophilus</i> Chester	Bergey et al., 1923
	<i>Escherichia ichthyosmius</i> Hammer	Bergey et al., 1923
	<i>Bacterium hydrophilum</i>	Weldin and Levine, 1923
	<i>Pseudomonas fermentans</i>	Wohlzogen-Kühr, 1932
	<i>Escherichia icteroides</i>	Pribham, 1933
	<i>Proteus ichthyosmia</i> Hammer	Bergey et al., 1934
	<i>Flavobacterium fermentans</i>	Bergey et al., 1934
	<i>Aeromonas liquefaciens</i>	Kluyver and van Niel, 1936
	<i>Proteus melanovogenes</i>	Miles and Halnan, 1937
<i>A. caviae</i>	<i>Vibrio jamaicensis</i>	Caselitz, 1955
	<i>Bacillus punctatus</i>	Zimmerman, 1890
	<i>Bacterium punctatum</i>	Lehmann and Neumann, 1896
	<i>Achromobacter punctatum</i>	Bergey et al., 1934
	<i>Pseudomonas caviae</i>	Scherago, 1936
	<i>Pseudomonas punctata</i>	Breed et al., 1948
	<i>Pseudomonas formicans</i>	Crawford, 1954
	<i>Aeromonas formicans</i> Crawford	Pivnick and Sabina, 1957
<i>A. sobria</i>	<i>Aeromonas punctata</i>	Schubert, 1967a, 1967b
	None	Popoff and Veron, 1976
<i>A. salmonicida</i>	<i>Bacillus</i> of trout pestilence	Emmerich and Weibel, 1894
	<i>Bacterium salmonicida</i>	Lehmann and Neumann, 1896
	<i>Proteus salmonicida</i>	Pribham, 1933
	<i>Necromonas achromogenes</i>	Smith, 1963
<i>A. media</i>	<i>Necromonas salmonicida</i>	Smith, 1963
	None	Allen et al., 1983
<i>A. veronii</i>	Enteric group 77	Hickman-Brenner et al., 1987
	DNA hybridization groups 8 and 10	Hickman-Brenner et al., 1987
<i>A. schubertii</i>	Enteric group 501	Hickman-Brenner et al., 1988
	DNA hybridization group 12	Hickman-Brenner et al., 1988
<i>A. eucrenophila</i>	<i>A. punctata</i>	Schuber and Hegazi, 1988

<sup>a</sup>Because of space limitations, some of these references are not included in the “Literature Cited,” this chapter; complete citations for some references can be found in Ewing et al. (1961).

<sup>b</sup>*B. ranicida* is considered a synonym only in the early literature.

system of classification of bacteria.” They attempted to make a more natural classification that was based on both morphological and physiological characteristics, and attempted to group similar organisms into the same family. However, it took many years to further define the genus *Aeromonas* and for it to be accepted as a genus. During the next 20 years, there was an intensive study of the existing species of *Aeromonas*, and several “new” ones were named. During this period many different classifications were proposed. It was very easy to find one or more phenotypic differences among a group of *Aeromonas* strains, but there was no agreement on how to draw species lines. The sixth edition of *Bergey’s Manual of Determinative Bacteriology* (Breed et al., 1948) still did not incorporate *Aeromonas* as a genus, and the species included in the genus today were classified in the genera *Pseudomonas* and *Bacterium*. The genus name *Aeromonas* did not appear in *Bergey’s Manual of Determinative Bacteriology* until the seventh edition in 1957 (reference is not an exact match Sniezesko, 1957), and there was little agreement on the number of species in the genus.

**THE 1960S AND THE 1970S** In the 1960s, there were a number of studies on nomenclature and taxonomy of *Aeromonas*, and it was during this decade that the definition of the genus was stabilized. In 1960, the first of the papers by Eddy suggested that the genus *Aeromonas* be redefined from the description given in the seventh edition of *Bergey’s Manual* (Breed et al., 1957). He proposed that the genus should contain only three species: *A. liquefaciens*, *A. salmonicida*, and *A. formicans*. Eddy (1962) and Eddy and Carpenter (1964) disagreed with the proposals of Ewing et al. 1961, and felt that the motile aeromonads could be separated into two species, *A. punctata* and *A. caviae*, based on the production of gas from glucose, acetylmethylcarbinol production, and gluconate oxidation. They further maintained that *A. punctata* should be the type species for the genus *Aeromonas*. They concluded that *A. punctata* was the legitimate designation for both *A. liquefaciens* and *A. hydrophila* and that the name *A. caviae* was the correct name for *A. formicans*.

In the eighth edition of *Bergey’s Manual* (Schubert, 1974) the genus *Aeromonas* was described as having three species: *A. hydrophila*, *A. punctata*, and *A. salmonicida*, with *A. hydrophila* as the type species. The descriptions were based on the earlier works by Schubert (1967a, 1967b, 1968, 1969a, 1969b, 1969c).

#### *Aeromonas* from 1979 to the Present

During this period, techniques that measure evolutionary distance were first used to study *Aero-*

*monas* strains. MacInnes et al. 1979 did the first DNA hybridization experiments with *Aeromonas* and concluded that the genus consisted of two main evolutionary lines: “a diverse group of motile aeromonads, and the genetically more homogeneous non-motile aeromonads, comprising the species *Aeromonas salmonicida*.”

Popoff et al. 1981 used DNA-DNA hybridization to study a group of motile *Aeromonas* strains from fish (37 strains), humans (10 strains), frogs (4 strains), and water (1 strain). The strains fell into at least seven different DNA hybridization groups, but several of the groups could be further subdivided on the basis of  $\Delta T^m$  values. The type strain of *A. hydrophila* was highly related to only two other strains, both isolated from fish. According to the *Bacteriological Code* this small group of three strains belong to the species *A. hydrophila* sensu stricto, (in a strict sense), because a species is defined in terms of its type strain and other closely related strains. Popoff et al. 1981 recognized two additional species, *A. caviae* (referred to as *A. hydrophila* biotype anaerogenes in the paper) and *A. sobria*. The study of Popoff et al. 1981 was the first to fully show the complex nature of the *A. hydrophila* group and to clearly define three of its species. Unfortunately, at least four of the groups defined by DNA hybridization could not be separated by simple phenotypic tests, and all three of the named species were heterogeneous because they contained one or more additional DNA hybridization group.

Fanning et al. 1985 obtained representatives of all the DNA hybridization groups defined by Popoff et al. 1981 and did additional DNA hybridization studies. They confirmed that all strains of *Aeromonas* are more closely related to each other than to species in other families, and found that the motile *Aeromonas* species could be divided into at least 10 different DNA hybridization groups.

#### Problems in Nomenclature and Classification of *Aeromonas*

There are two main problems in the nomenclature and classification of *Aeromonas*. The first is how best to indicate the divergence of *Aeromonas* from related organisms, a topic discussed in Chapter 156. The second problem concerns the nomenclature and classification of the individual *Aeromonas* species, which has been a confusing subject for many years. Although several studies have clearly indicated the evolutionary divergence of *Aeromonas* strains (MacInnes et al., 1979; Popoff et al., 1981), it has not been possible to find simple tests to define each of the DNA hybridization groups. However, several recent publications have helped to define small groups



of strains that can be differentiated. For example, Hickman-Brenner et al. 1987 defined *A. veronii* to include *Aeromonas* strains that are ornithine-decarboxylase positive and biochemically very similar to *Vibrio cholerae*. In a subsequent study, they named *A. schubertii*, including within this species a small group of strains that did not ferment D-mannitol (Hickman-Brenner et al., 1988). Similar studies are needed to better define all the DNA hybridization groups that have been identified in the motile *Aeromonas* group.

In addition to the problems in classification, there are also several problems in nomenclature. The biggest problem is that the name *Aeromonas caviae*, which is widely used in the literature, is a latter synonym of *Aeromonas punctata* and is thus illegitimate. This problem arose because an error was made in strain designation on the 1980 *Approved Lists of Bacterial Names*. There are several other nomenclatural problems, and a request to the Judicial Commission of the International Committee on Systematic Bacteriology will be needed to resolve them. Several different approaches for reporting strains of *Aeromonas* are given in the section on Identification.

## Habitats of *Aeromonas*

Strains of *Aeromonas* occupy a wide variety of ecological niches. Motile strain of the *Aeromonas hydrophila* group cause human extraintestinal infections and have also been implicated as causing diarrhea. They also cause a variety of diseases in both cold-blooded and warm-blooded animals. This group is also widely distributed in water and the environment. Most of the published reports on the *Aeromonas hydrophila* group have not subdivided them into currently recognizable species, and there are only a handful of studies that have differentiated them at the level of DNA hybridization group. In contrast, *A. salmonicida* occupies a limited ecological niche, producing furunculosis and bacteremia in fresh-water fish, particularly trout and salmon.

### Human Extraintestinal Infections

The first extraintestinal isolates of *Aeromonas* were reported by Bras et al. 1954 from a case of gas gangrene and independently by Hill et al. 1954 from a case of metastatic myositis. Hill et al. 1954 named their organism *Vibrio jamaicensis* and showed that it produced similar symptoms when pure cultures were ingested into rabbits, pigeons, and other animals. The first isolate from a blood culture was reported by Kjems (1955) soon after these initial reports. Many reports of extraintestinal isolates soon followed.

**MENINGITIS** *Aeromonas* is a rare cause of meningitis. Ellison and Mostow (1984) could find only five cases of meningitis in the literature when they did a survey in 1984. Two of four patients survived, and the fate of one was not mentioned. They also commented on the possible central nervous system (CNS) involvement in patients with *Aeromonas* bacteremia, and suggested that antibiotics chosen to treat bacteremia should be active against the bacterium and should provide adequate cerebrospinal fluid (CSF) concentrations.

**BACTEREMIA** *Aeromonas* occasionally causes bacteremia, particularly in immunocompromised individuals. Ellison and Mostow (1984) found fewer than 100 cases of *Aeromonas* bacteremia reported in the literature through 1984. A better estimate of the actual number of cases comes from the Communicable Disease Surveillance Center in England. In 1984 there were 45 cases of *Aeromonas* bacteremia in an unpublished surveillance report from England, Wales, and Ireland, and 13 of these (29%) were fatal cases. Most patients with *Aeromonas* bacteremia-septicemia have impaired host defenses that are usually the result of leukemia, other cancers, or liver disease (Ketover et al., 1973; Slotnick, 1970; von Graevenitz and Mensch, 1968). Bacteremia sometimes follows a wound infection that was originally contaminated with environmental water, or a surgical wound that was infected with *Aeromonas*. Bacteremia can also be primary, and result from intestinal tract colonization. Patients with leukemia or liver impairment are particularly susceptible to infection from the gut via the portal system. This is due to ulceration or mucosal breaks in the gastrointestinal (GI) tract due to toxic drugs or impairment of the hepatic reticuloendothelial system (Ellison and Mostow, 1984). *Aeromonas* bacteremia has also been reported in healthy individuals (Lynch et al., 1981; Scott et al., 1978) and may result from wound infection or gastrointestinal carriage.

Several species of *Aeromonas* have been isolated from blood. Janda and Brenden (1987) found that *A. sobria* predominated in 13 cases). *A. sobria* and *A. hydrophila* (3 of the 13 cases) were usually found in pure culture, but *A. caviae* (4 of 13 cases) was found in mixed culture. They commented that this correlated with the fact that *A. sobria* was generally more invasive for tissue than the other species. Of the newly described species *A. veronii*, *A. schubertii*, and *A. media*, only *A. schubertii* has been isolated from blood. Hickman-Brenner et al. 1988 reported that two of their eight isolates of *A. schubertii* were from blood.

**OTHER SITES** Strains of *Aeromonas* have been isolated from a wide variety of other infections. The most common source is wound infections, many of which are directly related to exposure to water (Joseph et al., 1979; Rosenthal et al., 1974; Slotnick, 1970; Stephen et al., 1975; von Graevenitz and Mensch, 1968). Numerous infected sites and sources of *Aeromonas* range from an alligator bite (Flandry et al., 1989) to urine.

**COMMUNITY-ACQUIRED VERSUS NOSOCOMIAL INFECTIONS** Washington (1972) reported that 19 of 27 *Aeromonas* strains were acquired in the community and the other eight were acquired in the hospital. Nine of the community-acquired infections were infected wounds which followed trauma, and eight of these were exposed to soil or water at the time of injury. Hospital-acquired infections due to *Aeromonas* appear to be increasing, but outbreaks are rarely reported. An interesting outbreak reported by Mellersh et al. 1984 involved 19 predominantly immunosuppressed patients who were infected or colonized with *Aeromonas*, three of whom acquired pneumonia.

**VIRULENCE FACTORS** Much work has been done on possible virulence factors in strains isolated from human patients with diarrhea, but much less has been done on the pathogenesis of extraintestinal infections. Strains of *Aeromonas* do not appear to be particularly virulent compared to other species, since most infections are in immunocompromised individuals. Ketover et al. 1973 concluded that two impor-

tant host defense mechanisms were the presence of specific opsonizing antibody and the bactericidal activity of neutrophils, and that these prevent invasive infections due to *Aeromonas*.

There is species-to-species and strain-to-strain variation in the virulence of *Aeromonas*. This has been shown for fish (reference is not an exact match De Figueiredo et al., 1977; Lallier et al., 1981; Wakabayshi et al., 1981) and mice, and it also appears to be true for humans. Table 2 compares three species of *Aeromonas* and lists some of the factors that have been mentioned as possible virulence factors. Strains of *Aeromonas* are known to make a wide variety of cytotoxins, exoenzymes, and other biologically active molecules. Endotoxin is probably a virulence factor in extraintestinal infections since it can cause a localized Schwartzman reaction and hemorrhagic lesions when injected into mice (Ketover et al., 1973).

Necrotizing hemorrhagic lesions have often been observed in *Aeromonas* infections, and these are probably related to one or more of the hemolysins-cytotoxins that many strains produce (Wretlind et al., 1971). Brook et al. 1985 injected strains subcutaneously and found that all nine strains of *A. hydrophila*, but only 3 of 15 strains of *A. sobria* caused abscesses. Many strains also produce an elastase which may be a virulence factor (Ljungh and Wadström, 1985. Janda et al. 1985 found that most of human clinical isolates *A. sobria* were virulent for mice ( $10^7$  cells, intraperitoneal injection) but that none of the *A. caviae* strains were virulent (Table 2).

Table 2. Pathogenic properties of the *Aeromonas hydrophila*, *A. caviae*, and *A. sobria* groups.<sup>a</sup>

Property	Result for phenotypic group <sup>b</sup>			Reference
	<i>A. hydrophila</i>	<i>A. caviae</i>	<i>A. sobria</i>	
Mouse virulence	Less	Less	More	Janda et al., 1985 Daily et al., 1981
Mouse mortality ( $10^7$ intraperitoneal)	- to +++++	-	+ to +++++	Janda et al., 1985
Human bacteremia	++	+	+++	Janda and Brenden, 1987
HEp-2 cell invasion	Less	Less	More	Watson et al., 1985
	+	-	++++	Lawson et al., 1985
Sensitivity to complement-mediated lysis by pooled human serum		++++		Janda et al., 1984
Extracellular enzymes	More	Fewer	More	Janda, 1985
Fish and animal disease	More	Less	More	
Abscesses in mouse model	++++		+	
Serum resistance	++	+	+++	Janda et al., 1984
Enterotoxigenicity	+	-	+	Daily et al., 1981; Burke et al., 1984
Role in human intestinal infections	+		+++	Dailey et al., 1981
Role in human intestinal infections	+	-	+++	Burke et al., 1984

<sup>a</sup>Each species contains two or more DNA hybridization groups.

<sup>b</sup>The results shown are our simplifications of the original data; the references should be consulted for more details. Adapted from reference is not an exact match Janada et al. (1983), Popoff (1984), and Sakazaki and Balows (1981).

## Human Infections: *Aeromonas* as a Cause of Diarrhea

There is a controversy on the etiological role of *Aeromonas* in diarrhea. Some laboratories routinely search for and report strains of *Aeromonas* as intrinsic enteric pathogens (*Salmonella* and *Shigella* are considered intrinsic enteric pathogens). Other authors are more cautious and warn that only some strains are likely to be pathogenic, a situation similar to that with *Escherichia coli* and *Yersinia enterocolitica*. One clear fact is that strains of *Aeromonas* occur in the feces of people both with and without diarrhea.

### History of *Aeromonas* Diarrhea

Environmental and animal isolates of *Aeromonas* date back to the 1890s but the first human isolate reported in the literature was in 1937 (Miles and Halnan, 1937). For many years thereafter there were few reports. Lautrop (1961) reported *Aeromonas* as the predominant organism in the feces of seven patients with acute intestinal problems and assigned a possible etiological role. Rosner (1964) reported a case of diarrhea that yielded *Aeromonas*, and this was probably the first report to give any evidence on the etiological role of *Aeromonas*. Today many authors report *Aeromonas* and assign it an etiological role.

### Epidemiology

Much has been written about the epidemiology of *Aeromonas* infections, but the situation is confused because the actual diarrheal disease may not have been caused by *Aeromonas*. Sporadic cases have been frequently reported, but documented outbreaks have been rare or non-existent. Most sporadic cases are community-acquired, although hospital-acquired cases have been reported. In industrialized countries, many cases occur during or just after travel to developing countries. Cases of *Aeromonas* diarrhea have been reported from most countries and are more common in the developing world. However, in Australia and in the state of Iowa there is an unusually high frequency among infants, which may be related to the presence of *Aeromonas* in well water or water-distribution systems. Strains of *Aeromonas* tend to occur more frequently in infants or in adults over 60 years of age (Agger et al., 1985; Burke and Gracey, 1986; Millership et al., 1983). Other risk factors include cancer, liver or biliary tract disease, and impaired immunity (Burke and Gracey, 1986), which are similar to the risk factors for extraintestinal infections due to *Aeromonas*.

**SOURCES** Most authors emphasize the widespread occurrence of *Aeromonas* in the environment, particularly in recreational and drinking water (Burke and Gracey, 1986; Burke et al., 1984a, 1984b; Millership and Chattopadhyay, 1985; Seidler et al., 1980). Several studies have indicated that most *Aeromonas* strains are isolated from humans during the warm months, when the organism is increasing in the environment. This seems to be true for both intestinal and extraintestinal infections. Food is also a likely source for *Aeromonas*, and food cultures have often been positive. Hunter and Broge (1987) found that 5% of ice cream samples in the United Kingdom were positive, and suggested that this may be due to post-processing contamination. Foods that come in contact with water during processing, such as washed vegetables, are also likely to be contaminated.

### Etiological Role in Diarrhea; Possible Pathogenic Mechanisms

**HUMAN VOLUNTEERS** The most damaging evidence against the etiological role of *Aeromonas* in human diarrhea was from the human volunteer studies of Morgan et al. (1985). Five strains were chosen on the basis of source and production of various suspected virulence factors such as hemolysin, cytotoxin, and enterotoxin (suckling mouse assay, rabbit ileal loop assay, cholera toxin cross-reactive factor). Three of the strains did not cause diarrhea or even colonize the volunteers. Strain 6Y from a healthy person in Bangkok, Thailand, colonized 11 of the 20 volunteers but there was diarrhea in only one, a person who had six unformed stools (with no fecal leucocytes) over 12 hours, with a short period of nausea, vomiting, anorexia, and malaise. He remained afebrile and did not develop a more progressive enteric illness. A biopsy of the small intestine was read as normal histologically. Strain 3647 from a diarrhea case in Perth, Australia, colonized three of 16 volunteers, but did not appear to cause illness. One volunteer had very mild diarrhea, but was culture-negative for the strain of *Aeromonas* that was given. This is probably the most important study that has dealt with the etiological role of *Aeromonas* in human diarrhea.

**INFANT (SUCKLING) MOUSE ASSAY** Several authors have found positive results in this assay, which was originally used for the heat-stable enterotoxin(s) of *E. coli*. Both viable cells and culture filtrates have been found to be positive (reference is not an exact match Pitaransi et al., 1982).

**SERENY TEST** This test for tissue invasiveness has been uniformly negative (Johnson and Lior, 1981; Ljungh and Kronevi, 1982; Pitarangsi et al., 1982).

#### COLONIZATION FACTORS AND HEMAGGLUTINS

Many bacterial species that cause diarrhea produce specific cell adhesins or other factors that allow them to colonize the intestine by attaching to cells of the intestinal mucosa. Agger et al. (1985) listed adhesion as a possible virulence factor for *Aeromonas*, and Daily et al. (1981) thought that pileation was involved. Strains of *Aeromonas* can attach to buccal (cheek) cells, apparently due to fimbriae (Atkinson and Trust, 1980; Daily et al., 1981). Attachment to HEp-2 cells or FL 100 intestinal cells does not appear to involve fimbriae. Strains also can attach to rabbit brush border cells and to red blood cells from a variety of animal species. Strains of *Aeromonas* produce two types of hemagglutinins. The soluble hemagglutinins are similar in some ways to those produced by *Vibrio cholerae* (Flandry et al., 1989; Notermans et al., 1986) except they lack protease activity. Soluble hemagglutinins are produced by *A. hydrophila*, *A. sobria*, and *A. caviae*.

#### Enterotoxins and Other Toxins

The exact mechanism by which strains of *Aeromonas* cause diarrhea (if they really do) is still unknown. Most of the evidence suggests that strains produce one or more toxins that eventually lead to diarrhea. Other studies suggest an invasive disease similar to *Salmonella* and *Shigella*, but this evidence is less compelling.

There is an extensive literature on the ability of *Aeromonas* to produce "enterotoxins" and other toxins. Much confusion has resulted because a single strain can produce several different proteins with biological activity. For example, most strains are hemolytic, and the hemolysin(s) produced during growth are toxic for animals and for animal cells grown in tissue culture. Strains have been reported incorrectly as enterotoxin producers based on the biological effects of these hemolysins. This type of error has been most common when a large number of strains has been screened in several different biological assays. The situation has been clarified by a more detailed study of a limited number of strains, and by subsequent purification and testing of the toxins produced (Chakraborty et al., 1984).

#### ENTEROTOXINS SIMILAR TO THOSE OF *E. COLI*

Many of the *Aeromonas* studies have utilized assays that were originally defined to study the enterotoxins of *E. coli*. Many of these assays

have been positive, but results from DNA probes now clearly indicate that there is no homology of *Aeromonas* with natural DNA probes for the heat-labile (LT<sup>P</sup>) and heat-stable enterotoxins (ST<sup>P</sup> and ST<sup>h</sup>) of *E. coli* (Pitarangsi et al., 1982).

**CHOLERA TOXIN** Some strains of *Aeromonas* produce a protein that is similar to cholera toxin. James et al. (1982) noticed the immunological cross-reactivity of their *Aeromonas* toxin and cholera toxin. Some strains of *Aeromonas* produce a protein that reacts in an ELISA assay (Chopra et al., 1986) or a commercial latex agglutination assay (Shimada et al., 1984) for cholera toxin. Schultz and McCardell (1988) found that nine of 12 *Aeromonas* strains reacted with an oligonucleotide-DNA probe for the A and B subunits of cholera toxin. This toxin was positive in the Y1 and CHO tissue culture assays and was heat stable. In immunoblots, the toxin appeared as three different bands with molecular weights of 89,900 (suspected to be the holotoxin), 37,000 (slightly larger than the A or A1 subunit of cholera toxin), and 11,000 (probably the B subunit). When the *Aeromonas* protein was incubated with antibody to cholera toxin, there was a 10-fold reduction in activity, and in an ELISA assay for cholera toxin, readings were equivalent to 3.5 to 100 mg of cholera toxin. The role of this cholera-like toxin in human diarrhea is unclear.

**TETRODOTOXIN** Tetrodotoxin is a potent neurotoxin that is produced by a variety of marine and land animals. Simidu et al. 1987 showed that a number of *Vibrio* and *Aeromonas* species appeared to produce tetrodotoxin or anhydrotetrodotoxin (a slightly different form of the toxin), and similar results were found by Tamplin et al. (1987). This new toxin is another potential virulence factor or cofactor.

**HEMOLYSINS** The hemolysin(s) produced by *Aeromonas* are potent toxins. Wretlind et al. (1971) purified two different hemolysins from an isolate of *A. hydrophila* and showed they had different isoelectric points. Both hemolysins were cytotoxic to HeLa and human fibroblast cells; lethal for mice; and caused dermonecrosis in rabbits. These biological activities would interfere with a number of enterotoxin assays. However, the hemolysins were destroyed by heating for 10 minutes at 56°C. Asao et al. (1984) found that 93% of *A. hydrophila* and 63% of *A. sobria* strains produced hemolysin that had cytotoxic and enterotoxic activity (positive reaction in the rabbit ileal loop). These results indicate that untreated supernatants of *Aeromonas* cultures will react in a variety of enterotoxin assays because hemolysin(s) is present.

**HEAT-STABLE, CYTOTONIC ENTEROTOXIN** This protein has been studied by several investigators, and it was recently cloned (Chakraborty et al., 1984). These authors found that two strains of *Aeromonas* from diarrhea patients in India had cytotoxic and hemolytic activity as well as enterotoxic activity. They cloned the enterotoxin gene of *Aeromonas* into *E. coli* and showed that the *E. coli* gene product caused elongation of CHO tissue culture cells and caused fluid accumulation in the rabbit ileal loop model and infant mouse model. Similar cloning experiments indicated that gene products of the hemolysin and cytotoxin genes were negative for fluid accumulation and that these three biological activities were located on three separate parts of the *Aeromonas* chromosome. They concluded that their toxin was a relatively heat-stable, cytotoxic enterotoxin clearly distinct from *E. coli* LT and ST and from hemolysin and cytotoxin.

### Does *Aeromonas* Cause Human Diarrhea?

Burke and Gracey (1986) summarized the role of *Aeromonas* in human diarrhea by saying:

Present evidence suggests that *Aeromonas* spp. are potential enteric pathogens with the risk of infection varying with age and geographical location but there remains the possibility that aeromonads could appear in diarrhoeal stools without being the aetiological agent. . . .

This is a good assessment of the evidence concerning *Aeromonas*, but it is also clear from the literature that many authors think that *Aeromonas* is an enteric pathogen. At present it seems clear that some strains of *Aeromonas* can cause diarrhea in some people. In a human volunteer study only one of five strains caused any diarrheal symptoms, and it caused a mild diarrhea in only one of 24 healthy human volunteers. A diarrheal disease is a function of both the host and the microbe. Future studies on the role of *Aeromonas* in diarrhea should focus on both these factors.

## ***Aeromonas* Diseases of Animals**

Both the *A. hydrophila* group and *A. salmonicida* are important causes of animal diseases. *A. salmonicida* is well known as a fish pathogen and the *A. hydrophila* group causes disease in fish, frogs, and several other animals.

### Furunculosis Caused by *A. salmonicida*

Furunculosis caused by *A. salmonicida* is a very important disease because it causes large economic losses of salmon and trout at commercial fish farms. Furunculosis is a communicable sep-

ticemic disease that is found in two different forms (McCarthy, 1977). In the acute form, there are few if any external signs, but there is a sudden onset and a high mortality. In the subacute form there are prominent focal muscle lesion or furuncles (hence the name furunculosis).

The disease has an extensive host range, but fish in the family Salmonida (which includes salmon and trout) are most susceptible, particularly brook trout and Atlantic salmon. Other fish that are affected either in the wild or on fish farms include cutthroat trout, Rocky Mountain white fish, brown trout, "sakuramasu" (*Onco-rhynchus gorbusha*), and many others.

The origin of the disease has been difficult to trace. Some authorities believe it was introduced into Europe in the 1880s with the import of rainbow trout, but others think it originated in Europe and was introduced to America with the brown trout (McCarthy, 1977). It was first introduced into the United Kingdom in 1911, and today it is found worldwide.

The disease is usually found in epidemic form (epizootic) rather than as isolated cases. It is more common in summer months as the water temperature rises. At fish farms it tends to be found where there is misuse of intensive rearing methods, usually by inexperienced personnel (McCarthy, 1977). This leads to stressed fish that are living under suboptimum environmental conditions.

A number of different names have been given to the "*Aeromonas*-like" organisms that have been isolated from fish with the disease. These include "Bacillus devorans" (Zimmerman, 1890), "Bacillus der Forellenseuche," *Bacterium salmonicida* (Emmerich and Weibel, 1894), *Bacterium devorans* (Zimmerman, 1890), *Haemophilus piscum* (Snieszko, 1964) *Necromonas* (Smith, 1963), *Necromonas achromogenes* (Smith, 1963), *A. salmonicida*, *A. salmonicida* subspecies *salmonicida*, and *A. salmonicida* subspecies *masoucida*. All of the earlier names are considered to be synonyms of *A. salmonicida*. Since the named subspecies of *A. salmonicida* are all highly related by DNA-DNA hybridization, it is clear that *A. salmonicida* is a species with variation in its phenotypic properties and host range in fish. There is little need therefore for maintaining three named subspecies.

### Diseases Caused by the *Aeromonas hydrophila* Group

In contrast to *A. salmonicida*, which primarily causes furunculosis in salmonid fish, species in the *A. hydrophila* group cause a wide variety of animal diseases, and are widespread in the environment, particularly water.

**RED LEG AND OTHER DISEASES OF FROGS** Red leg is an important disease because of the wide spread use of frogs in the laboratory. This septicemic disease in frogs is known as red leg because there are characteristic hemorrhages in the muscles of the legs (and abdomen) that cause the legs to have a distinctive red color (Russell, 1898). *A. hydrophila* has often been associated with outbreaks of this disease in frogs (Russell, 1898), particularly in laboratory populations (Gibbs, 1973).

**DISEASES OF FISH** The *A. hydrophila* group is also important in fish diseases, and can cause dramatic epidemics. Miller and Chapman (1976) reported 37,500 fish deaths during a 13-day period in one North Carolina lake. There have been similar reports for inland eutropic lakes (Hazen et al., 1978b; Shotts et al., 1972). One of the most commonly reported diseases is red sore disease of bass. However, other fish species can be affected including carp, cod (ulcer disease) (Larsen and Jensen, 1977), channel catfish (de Figueriedo and Plumb, 1977), shad (Haley et al., 1967), and centrarchid fish (Esch et al., 1976). Strains of the *A. hydrophila* group are almost always recovered from fish tissues, but the role of other microorganisms such as viruses and protozoa (Esch et al., 1976) must also be considered.

**OTHER ANIMAL DISEASES** There have been reports of other animal diseases, both in the wild and in laboratory populations. The first report of *A. caviae* (named *Pseudomonas caviae* in the original report) was by Scherago (1936, 1937) who described an outbreak of fatal septicemia in guinea pigs. The animals had been obtained in March 1935 from a local breeder and kept in the laboratory. Within a week, all 16 of the animals were dead. *A. caviae* was isolated from many tissues including liver, spleen, kidney, lung, and heart. The organism also caused a similar fatal infection in white mice but not in rabbits (Scherago, 1937).

Several other literature reports describe various aeromonads in animal diseases. These include "enterotoxin-producing" strains of *A. hydrophila* in piglet diarrhea (Dobrescu, 1978); ulcerative dermatitis and pneumonia associated with *A. hydrophila* in a bottle-nosed dolphin (Cusick and Bullock, 1973); hemorrhagic bacteremia in captive snakes that was transmitted by snakes mites (Camin, 1948); ulcerative stomatitis in captive snakes and lizards (Marcus, 1971); necrotic stomatitis in the desert tortoise (*Gopherus agassizi*) that develop pneumonia (Stroud et al., 1973); epidemic septicemia in reptiles at a zoo (Esterabadi et al., 1973; Heywood, 1968); eye infections and respiratory involve-

ment in laboratory lizards (Cooper et al., 1980); mortality in alligators (*Alligator mississippiensis*) in lakes and ponds where red sore disease was occurring in the fish population (Gorden et al., 1979); cutaneous ulcerations and septicemia in the aquatic African clawed frog *Xenopus laevis*; diarrhea and various other infections in birds (the etiology was not always investigated) (Panigrahy et al., 1981; Shane and Gifford, 1985; Shane et al., 1984); epidemic septicemia in commercial turkeys (Gerlach and Bitzer, 1971); various infections in animals raised for their fur such as mink, seals, and the blue fox (Dahle and Nordstoga, 1968); polyarthritis (Love and Love, 1984) and abortion (Wohlgemuth, 1972) in cows; septicemia in a dog (Pierce et al., 1973); and focal hepatic necrosis in young ferrets (Hiruma et al., 1986). The etiological role of *Aeromonas* was likely in many of these reports, but was less convincing or not investigated in some of the others.

## ***Aeromonas* in Water and the Environment**

Strains of *Aeromonas* have been isolated from a wide variety of water samples, and it is clear that members of this genus are primarily aquatic organisms. Many cases of infection or colonization in humans and animals can be traced to an exposure to water. In developing countries, people often drink untreated water, which may help to explain the frequent occurrence of *Aeromonas* in feces of people both with or without diarrhea. Many studies have focused on the distribution of *Aeromonas* in water and have tried to correlate a variety water chemistry parameters with counts of *Aeromonas* (Hazen et al., 1978b; Schubert, 1974).

### **Natural Waters**

Schubert (1967c) concluded that most of the *Aeromonas* strains in environmental water were not from human feces but represented the natural habitat of the organism. One of the largest surveys on the distribution of *Aeromonas* in water was done by Hazen et al. (1978b) in the United States. They sampled 147 natural aquatic habitats in 30 states and Puerto Rico and found that most were positive for *Aeromonas*. A few samples were negative, including water with a very high salinity (such as the Great Salt Lake), geothermal springs (45°C or higher temperatures), and three extremely polluted rivers. Of 147 samples, 135 were positive, including a wide variety of different habitats that varied in salinity, conductivity, temperature, pH, and turbidity. Saltwater samples had higher *Aeromonas* counts (a mean value of 746 organisms per ml)

than freshwater samples (a mean value of 130 organisms per ml), and lotic (rapidly moving) environments generally had higher counts than lentic (static) environments (mean values of 161 per ml versus 20 per ml). The highest count was 9,000 *Aeromonas* per ml at New Haven Harbor, Connecticut, a marine habitat with a salinity of 23 parts per thousand. Other samples with over 1,000 per ml included two creeks and two rivers. Although *Aeromonas* is not considered to be an obligate marine bacterium, the highest counts were found where marine ecosystems interface with fresh water, and it was found at all salinities except the most extreme (100% sea water). Similar results were found in Thailand, where 17 of 23 canals (klongs) were positive for *Aeromonas* (Pitarangsi et al., 1982). Versteegh et al. (International Workshop, 1986) studied the distribution of *Aeromonas* in different types of water. Spring water at its source and unpolluted ocean water were negative or had very low counts (2 per 100 ml), but flowing water further from its origin had much larger counts (400 to 1,000 per ml) which was probably due to sewage contamination. Recreational lakes had intermediate counts (10 to 100 per ml). Hazen and Fliermans (1979) studied the distribution of *Aeromonas* in a thermal gradient of effluent canal water at three nuclear reactors at the Savannah River Plant near Aiken, South Carolina. The highest number were found in water at temperatures of 21 to 33°C and none were found at temperatures higher than 45°C. These data are consistent with laboratory studies on the growth of *Aeromonas* in relation to temperature.

Sediment samples usually have much higher counts than the water column. In the Anacostia River near Washington, D.C., Seidler et al. (1980) found that sediment contained up to 400,000 *Aeromonas* per gram compared to 100–300 per ml in the water column. However, in a water reservoir used for cooling at a nuclear reactor, Hazen (1979) found higher counts in the water column than in the sediment. Higher counts are usually present where there is a high concentration of particulate or dissolved organic matter (Hazen, 1979). The counts of *Aeromonas* in environmental water increased as the temperature rose and dropped dramatically during winter (Seidler et al., 1980). *Aeromonas* counts were also high in sewage, and in household and industrial effluents, which may be important sources of *Aeromonas* in the environment.

**DRINKING WATER** *Aeromonas* is often found in drinking water (Meeks, 1963; Schubert, 1967c) and consequently in sinks, tubes, drain pipes, and household effluents (Schubert, 1967c). Well

water in both developing and industrialized countries also contains *Aeromonas* (von Graevenitz and Mensch, 1968). reference is not an exact match Pitaransi et al. 1982 found that 15 of 15 water jugs (ongs) in Thailand were positive for *Aeromonas*. In one case, water from an artesian well contaminated chicken carcasses as they were being washed (Eddy, 1960). The frequent presence of *Aeromonas* in drinking water must be considered in all studies that attempt to define its role as an enteric pathogen.

**HOSPITAL ENVIRONMENT** Washington (1972) rarely found *Aeromonas* in hospital environmental surveys. However, Rafferty and Pancoast (1984) noted *Aeromonas* contamination on bedpans, toilet flush handles, telephones, and dictaphones. The contamination was very light and was thought to be of little significance. *Aeromonas* has also been found in the hospital water supply (Meeks, 1963; Conn, 1964), and even in chlorinated hospital water (Millership and Chattopadhyay, 1985). It was found in 71% of the samples that contained *E. coli* and 19% of those that did not contain *E. coli* (Millership and Chattopadhyay, 1985).

**OTHER AQUATIC SAMPLES** *Aeromonas* has been isolated from raw sewage, treated sewage, activated sludge (Neilson 1978), the mud of sink siphons in waste water drainage systems (Schubert, 1967c), swimming pools (von Graevenitz and Mensch, 1968), and many other aquatic samples.

**OTHER SAMPLES** Decomposing plant material and fecal material from animals usually contain large numbers (Hazen, 1979). Sanyal et al. 1987 found *Aeromonas* in 98 of the 100 tropical aquaria sampled.

**THE COLIFORM CONCEPT—CONFUSION WITH *E. COLI*** Some strains of *Aeromonas* ferment lactose at 37°C with the production of gas. For this reason they can be considered to be “coliforms,” and thus can be an important source of error in measuring the bacteriological quality of water. Leclerc and Buttiaux (1962) found that 30% of 9,036 drinking water samples were positive for “fecal-coliform” positive strains of *Aeromonas*, and if further testing had not been done these would have falsely indicated that the sample was positive in the fecal coliform test. A positive-oxidase test easily differentiates *Aeromonas* from *E. coli*, as would the more specific fecal-coliform test in many instances. The widespread distribution of *Aeromonas* in both drinking and environmental water must be considered in bacteriological analysis.

**DISTRIBUTION OF *AEROMONAS* SPECIES IN WATER** Most studies have not differentiated the *Aeromonas* species or DNA hybridization groups. However, Versteegh et al. (International Workshop, 1986) concluded that *A. hydrophila* predominates in spring water, with about 80% positive. *A. caviae* predominates in marine samples with about 60% positive. *A. sobria* predominates in recreational lakes with about 50% positive. All three species are found in flowing water polluted with sewage.

## Isolation of *Aeromonas*

The *A. hydrophila* group occurs in a wide variety of human clinical and environmental specimens, and most strains grow well at 35–37°C as well as 20–25°C. Much of the literature on *Aeromonas* isolation is about this mesophilic group. In contrast, *A. salmonicida* does not grow at 37°C and is usually found only in diseased fish.

### Isolation of *A. salmonicida*

Swabs of the fish surface, lesions, or tissue removed at autopsy can be streaked on nutrient agar containing 0.1% tyrosine or phenylalanine, and incubated at 22°C. Strains of *A. salmonicida* grow at this temperature and most typical strains produce a soluble brown pigment.

### Isolation of the *A. hydrophila* Group

Some authors feel that *Aeromonas* is an enteric pathogen, and that a selective medium should be incorporated for the isolation of all strains, as is done for *Salmonella* and *Shigella*. We and others feel that only certain strains of *Aeromonas* are pathogens, and that the use of selective media is not indicated because small numbers of *Aeromonas* would be isolated. These strains presumably would not be enteric pathogens, but would probably represent transient gut colonization or merely the passage of organisms ingested from food or drinking water. Both approaches are described below, and the reader is encouraged to use the approach that best fits the laboratory's particular needs. Most isolation procedures used in the clinical microbiology laboratory for extraintestinal specimens should be effective in isolating *Aeromonas* since it grows well on most plating media.

**BLOOD AGAR** Most extraintestinal specimens are plated on blood agar, and this medium is also recommended for intestinal (stool) specimens. Colonies of *Aeromonas* are 2–3 mm in diameter after 24 h at 35–37°C and many strains are hemolytic. Individual colonies can be tested

for their oxidase reaction (Kovacs method). Oxidase-positive hemolytic colonies (that do not have the characteristic color and odor of *Pseudomonas aeruginosa*) are more likely to be *Aeromonas* than other species. A sweep of several hundred colonies can also be done to detect oxidase-positive colonies mixed with oxidase-negative colonies. If the sweep is oxidase-positive (either weakly or strongly), individual colonies can then be tested (Janda et al., 1984b).

**MACCONKEY AGAR** Almost all strains grow on MacConkey agar and most grow on SS agar (Nygaard et al., 1970). Different strains of *Aeromonas* can vary in appearance on this medium from bright red with precipitated bile around the colony (indistinguishable from *E. coli*) to light pink to colorless. This is because strains of *Aeromonas* vary in the speed and degree of lactose fermentation. Many strains produce colorless (lactose-negative) colonies and probably would be picked as suspect *Salmonella* or *Shigella*.

**OTHER PLATING MEDIA** Two other plating media often used for fecal cultures are somewhat selective for *Aeromonas* even though they were designed for the isolation of other organisms. CIN medium (cefsulodin, irgasan, novobiocin) for *Yersinia* isolation is routinely used in many laboratories and will often select for *Aeromonas* (International Workshop, 1986). Similarly, Misra et al. (1989) showed that *Aeromonas* was often isolated on Butzler's *Campylobacter* selective agar. This medium was compared with sheep blood agar containing 30 µg of ampicillin per ml for the isolation of *Aeromonas* from diarrheal stool cultures. Twenty-six of the specimens were positive for *Aeromonas* on both media; 29.6% were positive on Butzler's *Campylobacter* agar only and 43.7% were present only on blood agar plus ampicillin. Other selective media for *Campylobacter* (Skirro's formulation and Blazer's) contain trimethoprim and polymyxin B and are not suitable for *Aeromonas* isolation because most strains are inhibited by these agents.

### Special Media For *Aeromonas*

A number of different media have been described for the isolation of *Aeromonas*, and there have been several evaluations and reviews (von Graevenitz and Bucher, 1983). There is some confusion in the literature on the value of the many *Aeromonas* media, probably due to differences in the populations tested and the fact that different *Aeromonas* species and DNA hybridization groups have been used. We will describe in detail only those media that have been used frequently or that are commercially available.



### Bile Salts-Brilliant Green Agar for *Aeromonas* and *Plesiomonas*

Most media were designed to isolate either *Aeromonas* or *Plesiomonas*; one exception is bile salts-brilliant green agar, which was formulated to select for both genera. This medium was described by Millership and Chattopadhyay (1984). It contains bile salts and brilliant green, which should inhibit many enteric and other bacteria.

Nutrient broth no. 2 (Oxoid)	25 g
Bile salts	5 g
Brilliant green, 0.05% solution	1 ml
Agar	12 g
Water	1 liter

Adjust the pH to 7.0. Dissolve all the ingredients by heating to boiling; do not autoclave. Cool to 45–50°C and pour into plates. Streak the specimen onto the plate and incubate at 37°C for 18 h. Flood the plate with oxidase reagent and immediately pick and subculture oxidase-positive (purple) colonies, and further identify them.

### Dextrin-Fuchsin-Sulfite Medium (*Aeromonas* Differential Agar)

This medium is one of the first isolation media for *Aeromonas*, and it was originally described by Schubert (1967a, 1967b). It contains dextrin which is metabolized by *Aeromonas* but not by most other enteric bacteria except *Enterobacter*. Dextrin catabolism is indicated by an aldehyde formed with fuchsin which has been reduced by sulfite. This latter reaction is known as the “Endo principle” and has occasionally been used in the formulation of enteric media. The medium can be modified by the addition of various compounds to inhibit Gram-positive organisms and can also be used to isolate *Enterobacter agglomerans* (Bucher and von Graevenitz, 1982).

The following formula is the one given by E. Merck, Darmstadt, Germany, which at one time produced and sold this medium as “*Aeromonas* Differential Agar,” catalog no. 15105.

Peptone (from casein)	10 g
Meat extract	3 g
Sodium chloride	5 g
Dextrin	15 g
Sodium sulfite	1.6 g
Fuchsin	0.25 g
Sodium phosphate (Na <sub>2</sub> HPO <sub>4</sub> )	7.75 g
Agar	13 g
Water	1 liter

Adjust the pH to 7.5 ± 0.2. Suspend the ingredients in the water and boil until they dissolve completely. (Merck indicates that it may be helpful to first suspend them in 50 ml of a 5% aqueous solution of dioxane to better dissolve the fuchsin.) Autoclave at 121°C for 15 min. Cool to 45–50°C and pour into plates.

Inoculate the specimen onto the medium, and incubate at either 30 or 37°C. Further incubation may be required and should be done at room temperature. Colonies of *Aeromonas* are moist and red because they metabolize the dextrin. Colonies of most species of Enterobacteriaceae and *Pseudomonas* do not metabolize the dextrin and are colorless. Some strains of *Enterobacter* and *Klebsiella* metabolize dextrin and also form red colonies, but they can be differentiated after subculture and oxidase testing. (The manufacturer says that the plate can be sprayed with or flooded with oxidase reagent to detect

oxidase-positive colonies.) Suspicious red colonies are picked and further identified. The manufacturer says that the vibriostatic compound 0129 can be added to the medium to inhibit *Vibrio* species.

### Rimler-Shotts Medium

This medium (Shotts and Rimler, 1973) was designed for the isolation of *Aeromonas* from water and animals. It contains novobiocin and deoxycholate to inhibit Gram-positive organisms and vibrios. It contains maltose, which is almost always fermented by *Aeromonas*, and the pH indicator bromthymol blue. It contains L-lysine and L-ornithine which are often decarboxylated by enteric bacterial to give alkaline products (in 1973 *Aeromonas* was thought to be arginine-positive, but lysine-negative and ornithine-negative). It also contained thiosulfate and a ferric salt to indicate H<sub>2</sub>S production by organisms such as *Citrobacter*. This medium has been used in a number of studies on the distribution of *Aeromonas* in the environment.

L-Lysine hydrochloride	5 g
L-Ornithine hydrochloride	6.5 g
Maltose	3.5 g
Sodium thiosulfate	6.8 g
L-Cysteine hydrochloride	0.3 g
Bromthymol blue	0.03 g
Ferric ammonium citrate	0.8 g
Sodium deoxycholate	1.0 g
Novobiocin	0.005 g
Yeast extract	3 g
Sodium chloride	5 g
Agar	13.5 g
Water	to 1 liter

Suspend all of the ingredients in the water and adjust the pH to 7.0. Heat to boiling and boil gently for 1 min. Cool to 45°C and pour into petri dishes. Refrigerate until used.

Plate the sample onto the medium and streak to obtain isolated colonies. Incubate at 37°C and observe after 20 h but no later than 24 h. Typical strains of *Aeromonas* are yellow (maltose-positive) without a black center. Other enteric bacteria had a variety of other appearances.

We suspect that lysine-positive and ornithine-positive strains of *Aeromonas* may not have the typical strong yellow color because of alkaline products produced during decarboxylation of the amino acid.

### *Aeromonas* Agar

This is one of the few media for the isolation of *Aeromonas* that is commercially available in a complete form. It contains sodium deoxycholate to inhibit Gram-positive organisms and sodium citrate to inhibit other enteric bacteria. D-Xylose is present as a differential sugar. Most *Aeromonas* strains do not ferment this sugar. Sodium thiosulfate and ferric citrate are included to indicate H<sub>2</sub>S production.

<i>Aeromonas</i> agar base (see below)*	47 g
Water	100 ml

pH 7.0 ± 0.2 at 25°

\*Also available as a dehydrated powder from Gibco; catalog no. 152-0149M.

Formula for *Aeromonas* agar base:

Peptone 140 (pancreatic digest of casein)	5 g
Beef extract	5 g

D-Xylose	10 g
Sodium chloride	2.5 g
Ferric ammonium citrate	1 g
Sodium citrate	5 g
Sodium deoxycholate	1.5 g
Sodium thiosulfate	5 g
Neutral red	0.025 g
Agar	12 g

Suspend the powder in the water. Heat to boiling to dissolve. Allow to simmer for 20 seconds. Do not autoclave or overheat. Cool to 45–50°C and pour onto plates.

Inoculate the specimen onto the medium and streak to get isolated colonies. Examine the plate and pick xylose-negative colonies as suspect *Aeromonas* for further testing. An alternative is to enrich the specimen in alkaline peptone water first, and then plate it onto the *Aeromonas* agar. Colonies of *Aeromonas* appear as moist pale or colorless colonies about 2 mm in diameter. Strains of *A. sobria* have a slight golden sheen when growth is confluent.

### Blood Agar Plus Ampicillin

Most strains of *Aeromonas* from clinical specimens are resistant to 10–30 µg of ampicillin; this led to the formulation of this medium which has often been used in clinical microbiology laboratories. Some *Aeromonas* strains will be inhibited by this concentration of ampicillin, but the majority will grow.

Trypticase soy agar*	40 g
Water	950 ml
Sheep blood	50 ml
Ampicillin**	10 mg

\*Other blood agar bases can also be used.

\*\*Other concentrations of ampicillin have been used, including 15 µg/ml (Agger, 1986) and 30 µg/ml.

Suspend the trypticase soy agar in the water and heat to boiling to dissolve. Autoclave at 121°C for 15 min. Cool to 45–50 °C and add the sheep blood and the ampicillin, and pour into plates. Store in the refrigerator in sealed bags until needed. Strains of *Aeromonas* will usually grow and be strongly beta-hemolytic on this medium. Suspicious colonies can be tested for their oxidase reaction.

Prepared plates are available from Remel and contain 10 µg/ml of ampicillin (catalog no. 01–226).

### DNase Agar Plus Ampicillin and Toluidine Blue

von Graevenitz and Zinterhofer (1970) described this medium for the isolation of *Aeromonas*. They added ampicillin and toluidine blue to DNase test agar, which is commercially available, and found the medium useful in isolating *Aeromonas* from stool cultures. It should also be useful for other types of specimens.

DNase test medium	42 g
Toluidine blue solution (1%)	10 ml
Water	990 ml
Ampicillin	30 mg

Suspend the DNase test medium in the water. Add the toluidine blue solution. Heat to boiling to dissolve the agar. Autoclave at 121°C for 15 min and cool to 45°C. Aseptically add a solution of ampicillin to give a total of 30 mg. Pour into petri dishes; let cool and store in the refrigerator.

Streak a plate with the sample to obtain isolated colonies. Incubate at 35–37°C for 16–24 h, and look for typical colonies. Colonies of *Aeromonas* grow on the medium and have a large pink zone around them because of DNA hydrolysis. Strains of Enterobacteriaceae that normally occur in feces are usually inhibited or are DNase-negative.

### MacConkey Agar Plus Ampicillin and Tween-80

This medium is made by adding ampicillin and Tween-80 to MacConkey agar, and is commercially available in plates from Remel (catalog no. 01–560). Strains of *Aeromonas* hydrolyze Tween-80 and have an opaque zone around them. Most Enterobacteriaceae are inhibited or are negative for Tween-80-hydrolysis.

### Pril-Xylose-Ampicillin Agar

This medium was described in 1978 by Rogol et al. (1979) and contains ampicillin and pril (a quaternary ammonium detergent) to inhibit Gram-positive and other enteric bacteria. It contains D-xylose, which is not fermented by most strains of *Aeromonas*, which produce colorless colonies. The medium also prevents swarming of *Proteus* strains.

Nutrient agar	8 g
D-Xylose	10 g
Phenol red	0.025 g
Ampicillin	30 mg
Pril*	0.2 g
Water	1000 ml

\*Obtained from Böhme Fettchemie, GmbH, Düsseldorf, Germany.

### OTHER ISOLATION MEDIA FOR *AEROMONAS*

Other media that have been mentioned as being useful for the isolation of *Aeromonas* include: alkaline peptone water (Millership and Chattopadhyay, 1984), xylose-deoxycholate-citrate agar (Millership and Chattopadhyay, 1984), bile salts-brilliant green agar (Millership and Chattopadhyay, 1984), and starch-ampicillin agar (Palumbo et al., 1985).

### Use of Selective Media

We feel that selective media have a limited role in the routine clinical microbiology laboratory, but can be used in laboratories specifically evaluating the role of *Aeromonas* in diarrhea or isolating *Aeromonas* from the environment. There is no universal agreement that a single *Aeromonas* medium is superior to the others for a given application.

## Identification of *Aeromonas*

Strains of *Aeromonas* are small Gram-negative rods that have no outstanding morphological features. They usually have a single polar flagellum (Fig. 1), but flagellar strains are not normally used in identification. The *A. salmonicida* and *A. hydrophila* groups are very different in their phenotypic characteristics. *A. salmonicida* is likely to

Fig. 1. Cellular morphology of *A. hydrophila*. (A) is redrawn from a photomicrograph of a flagella stain given in Fig. 1 of Ewing et al. (1961). With flagellar stains, the flagella appear much larger than actual size. (B), (C), and (D) are redrawn from some electron micrographs in Plate 1 of Eddy (1960). Bars = 1  $\mu$ m.

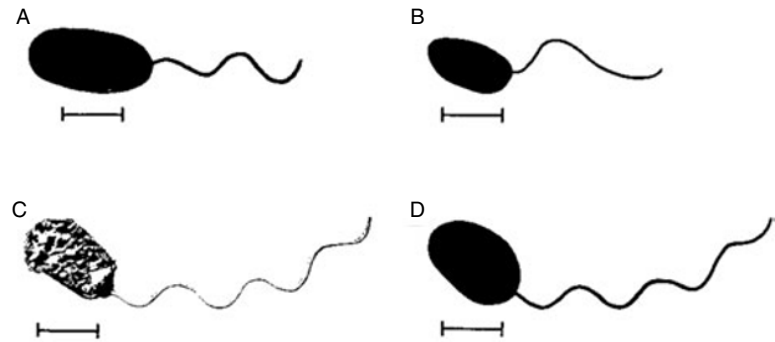


Table 3. Differentiation and properties of the species groups and species of the *Aeromonas hydrophila* group that occur in human clinical specimens.

Test or property	DNA hybridization group <sup>a</sup>			Species <sup>b</sup>	
	<i>A. hydrophila</i>	<i>A. caviae</i>	<i>A. sobria</i>	<i>A. veronii</i>	<i>A. schubertii</i>
Voges-Proskauer	+	-	V	(+)	-
Gas production from D-glucose	+	-	+	+	-
KCN, growth in	+	+	-	V	-
Salicin fermentation	+	+	-	+	-
Esculin hydrolysis	+	+	-	+	-
Ornithine decarboxylase (7-day)	-	-	-	+	-
Utilization of					
L-Arginine	+	+	-		
L-Histidine	+	+	-		
L-Arabinose	+	+	-		
H <sub>2</sub> S production from L-cysteine	+	+	-		
Elastase production	+	-	+		
Citrate utilization (Kauffmann-Peterson method)	-	+	-		

Symbols: +, most strains are positive; (+), many strains are positive; -, most strains are negative; V, variable and of limited value in differentiation.

<sup>a</sup>*A. hydrophila*, *A. caviae*, and *A. sobria* are each composed of two or more DNA hybridization groups. The data for these three species were adapted from Janda et al. (1983), Popoff (1984), and Sakazaki and Balows (1981). The media, incubation times, and temperatures vary from author to author.

<sup>b</sup>The data for *A. veronii* and *A. schubertii* are based on stains studied in our own laboratory (36°C, 2 days incubation).

be isolated only by those who work with fish diseases.

Strains of the *A. hydrophila* group grow both in the presence and absence of oxygen, ferment glucose (sometimes with gas production), are oxidase- and catalase-positive, grow well on most enteric plating media, do not require Na<sup>+</sup> for growth, are not inhibited by the vibriostatic compound 0129, and are usually resistant to penicillins. The test results useful for identification and differentiation are given in Table 3. Some of the tests results are dependent on media and temperature.

There is considerable variability in the biochemical reactions of the motile *Aeromonas* species (Ewing et al., 1961; Janda, 1985). Some strains with unusual reactions, such as those that are mannitol negative or ornithine decarboxylase-positive, were eventually recognized as new species, but other atypical strains of *Aeromonas* have been described (Hansen and Glupczynski, 1984) and may represent new species or new biogroups of existing species. Some strains of

*Aeromonas* ferment lactose rapidly at 37°C, and Leclerc and Buttiaux (1962) found that this was an important source of error (30% of 9,036 samples) in counts of coliform bacteria in drinking water. Occasionally, strains of the *A. hydrophila* group produce brown pigment (Ross, 1962), a property usually associated with *A. salmonicida*.

### Oxidase Test

McGrath et al. (1977) found that an isolate from bile was oxidase-positive (Kovacs method with tetramethylphenylenediamine dihydrochloride) when it was grown on five different nonselective media, but was oxidase-negative when grown on XLD agar, EMB agar, SS agar, or brilliant green agar. The next six strains of *Aeromonas* isolated in the laboratory were oxidase positive on all media tested. In a subsequent study, it was found that eight of 100 strains of *Aeromonas* had this medium-dependent reaction, and all eight were lactose fermenters on enteric isolation media (Overman et al., 1979). Other lactose-positive

strains of *Aeromonas* did not have this medium-dependent oxidase reaction, nor did any of the lactose-negative strains.

### Decarboxylase Reactions

Since the decarboxylase reactions are important in the identification of *Aeromonas*, Altwegg et al. (1987) compared five different media for the determination of lysine and ornithine decarboxylase and arginine dihydrolase. There was considerable variation in the five media and also variation depending on the temperature of incubation and time at which the reactions was read.

## Identification in the Clinical Microbiology Laboratory

Hemolytic colonies of “enteric”-type organisms on sheep blood agar should be considered as being suspicious for *Aeromonas*. Oxidase testing quickly rules out hemolytic strains of Enterobacteriaceae, but does not rule out *Vibrio cholerae* non-O1, which is another hemolytic oxidase-positive species that can occur in clinical specimens. Biochemical testing is required to complete the identification, and to differentiate some of the species in the *A. hydrophila* group (Table 3). This testing can be done in conventional tubed media or in commercial identification systems. Commercial systems are usually good in identifying a strain as belonging to the *A. hydrophila* group but they vary in ability or attempts to split this broad group. Some systems report the three main subgroups *A. hydrophila*, *A. sobria*, and *A. caviae*; others report *A. hydrophila* (in a broad sense, meaning the whole group); the *A. hydrophila* group; or simply *Aeromonas*. Several of the commercial identification systems that include *Aeromonas* have been evaluated by a number of

authors (Altwegg and Zollinger-Iten, 1987; Greene et al., 1984; Overman et al., 1979; Teska et al., 1989; Toranzo et al., 1986).

Strains of the *A. hydrophila* group can be confused with *V. cholerae*, *V. fluvialis*, and *V. furnissii*. However these organisms are quite distinct, as can be shown with complete testing (Table 4). Strains of *Aeromonas* grow in nutrient broth with no added NaCl, which is in contrast to most species of *Vibrio* which require Na<sup>+</sup>. *A. veronii* has the same decarboxylase pattern as *V. cholerae* (lysine-positive, arginine-negative, ornithine-positive), but these two species can be differentiated with complete biochemical testing or antibiotic susceptibility testing (Table 4).

## Screening Tests

In large environmental surveys it is often desirable to screen colonies and discard those not likely to be *Aeromonas*. Kaper et al. (1979) suggested the use of “AH medium” as a simple way to identify *Aeromonas* cultures, which reacted differently from many other common organisms (Table 5). Interestingly, Toranzo et al. (1986) found that the result for several of the tests in this screening medium did not agree with the results when the test was done in a standard tube test.

### AH Medium

Proteose peptone	5 g
Yeast extract	3 g
Tryptone	10 g
L-Ornithine hydrochloride	5 g
D-Mannitol	1 g
myo-Inositol	10 g
Sodium thiosulfate	0.4 g
Ferric ammonium citrate	0.5 g
Bromocresol purple	0.02 g
Agar	3 g
Water	1 liter

Table 4. Differentiation of the arginine positive species of *Vibrio* (*V. damsela*, *V. fluvialis*, and *V. furnissii*) and comparison with *Aeromonas*.

Test or property	<i>V. damsela</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>Aeromonas</i>
Growth in nutrient broth with:				
No added NaCl	–	–	–	+
1% NaCl	+	+	+	+
Voges-Proskauer	+	–	–	V
Simmons citrate	–	+	+	V
Fermentation of:				
D-Galacturonic acid	–	+	+	–
L-Arabinose	–	+	+	V
D-Mannitol	–	+	+	+
Sucrose	–	+	+	(+)
Gas production during fermentation	V	–	+	V

Symbols (all data are for reactions within 2 days at 35–37°C unless otherwise specified): +, 90–100% positive; (+), 75–89.9% positive; V, 25.1–74.9% positive; (–), 10.1–25% positive; –, 0–10% positive.

Table 5. Reactions and properties of *Aeromonas* and other bacteria in the medium of Kaper et al. (1979).

Species	Reaction at:		Motility	H <sub>2</sub> S	Indole
	Top	Butt			
<i>Aeromonas</i>	K	A	+	–	+
<i>Klebsiella pneumoniae</i>	A	A	–	–	–
<i>K. oxytoca</i>	A	A	–	–	+
<i>Escherichia coli</i>	K	K or A	V	–	+
<i>Salmonella</i> sp.	K or A	K or A	+	+	–
<i>Enterobacter</i> sp.	K or N	K or N	+	–	–
<i>Proteus</i> sp.	Red	K or A	+	V	V
<i>Yersinia enterocolitica</i>	K or N	K or N	–	–	V
<i>Citrobacter</i> sp.	K	A or K	+	+	–
<i>Serratia</i> sp.	N or K	N or K	+	–	–

Symbols: K, alkaline reaction; A, acid reaction; N, a bleached neutral color due to decolorization of the indicator; +, 90% or more are positive; –, 10% or less are positive; V, strain to strain variation (11–89% positive).

Suspend the ingredients in the water. Adjust to pH 6.7, and heat to boiling to dissolve the agar. Dispense 5 ml into 13 by 100 mm screw-cap tubes and autoclave at 121°C for 12 min. Cool and store in the refrigerator.

With a straight wire, pick colonies that are suspicious for *Aeromonas*, and stab into the medium going all the way to the bottom of the tube.

### Other Screening Tests

Other useful tests that can be used for *Aeromonas* screening include: ampicillin resistance, starch or dextrin fermentation, and a positive reaction for DNase and Tween-80 hydrolysis. As previously mentioned, these reactions are often incorporated in selective-differential media for *Aeromonas*.

### Serotyping

Until recently there was no complete antigenic schema for serotyping *Aeromonas*. Ewing et al. (1961) established a preliminary serotyping system for *Aeromonas* by defining 12 somatic (O) and 9 flagellar (H) antigens. For many years there was little interest in further developing the schema. However, Sakazaki and Shimada (1984) described a schema with 44 O antigens that was useful for typing all the species included in the *A. hydrophila* group. The schema was based on antisera produced in rabbits with O antigen preparations (heated cultures). Unfortunately, most of the sera contained “rough-agglutinins” and required absorption with strain 1281–87 (a rough strain) before they could be used in routine typing. Some of the O antigens of *Aeromonas* were similar or identical to O antigens of *V. cholerae* or *Plesiomonas shigelloides*. This schema and its subsequent refinements should be useful in epidemiological and ecological studies and may help to define the etiological role of *Aeromonas* in diarrhea. Several other typing methods have been used to differentiate strains

below the level of species, but these are more specialized or difficult to do routinely.

### Antibiotic Susceptibility

Most strains of *Aeromonas* are susceptible to the antibiotics chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, gentamicin, amikacin, nitrofurantoin, tobramycin, and third-generation cephalosporins. Most strains are resistant to penicillin, ampicillin, carbenicillin, methicillin, erythromycin, clindamycin, and vancomycin. About 50% of the strains are also resistant to piperacillin, mezlocillin, and second-generation cephalosporins (cefoxitin and cephmandole) (Motyl et al., 1985). The susceptibility patterns of *A. hydrophila*, *A. sobria*, and *A. caviae* are very similar. Some strains of *A. caviae* apparently do not produce  $\beta$ -lactamases and are sensitive to ampicillin (Richardson et al., 1982). Strains with transferable antibiotic resistance have been described, particularly in developing countries, but they are relatively rare in human clinical specimens. R factor-containing strains can also be important in the fish farming industry because antibiotic usage is a strong selective pressure for the development of resistance (Popoff and Devaine, 1971).

### Identification of *Aeromonas salmonicida* (Sakazaki and Balows, 1981)

Strains isolated from diseased fish are probably *A. salmonicida*, if they are Gram-negative, fermentative, rod-shaped, nonmotile, oxidase-positive, grow at 25°C but not at 36°C, and produce a soluble brown pigment. The identification can be confirmed with a complete set of biochemical reactions and perhaps other testing. Strains of *A. salmonicida* usually produce extracellular enzymes such as gelatinase, amylase, lecithinase, and lipase. They ferment D-glucose, usually with slight gas production, and most strains ferment

Table 6. Differentiation of the three subgroups (subspecies) of *Aeromonas salmonicida*.

	Subgroup		
	salmonicida	achromogenes	masoucida
Brown soluble pigment	+	-	-
Gas production from D-glucose	+	-	+
Esculin hydrolysis	+	-	+
D-Mannitol fermentation	+	-	+
L-Arabinose utilization	+	-	+
Indole production	-	+	+
Sucrose fermentation	-	+	+
H <sub>2</sub> S production from L-cysteine	-	-	+
Voges-Proskauer	-	-	+

Adapted from Popoff (1984).

Table 7. Phage typing scheme for *Aeromonas salmonicida*.

Phage type	Lysis by phage:								Percent of strains lysed
	31	32	25	51	56	65	63	29	
1	+	+	+	+	+	+	+	+	10
2	+	+	-	+	+	+	+	+	1
3	+	+	+	+	+	+	+	-	1
4	+	+	+	+	+	+	-	+	3
5	+	+	+	+	+	+	-	-	1
6	+	+	+	+	+	-	-	-	59
7	+	+	+	+	-	-	-	-	3
8	+	+	+	-	-	+	+	+	2
9	+	+	+	-	-	-	-	+	1
10	+	+	+	-	-	+	-	-	2
11	+	+	+	-	-	-	-	-	13
12	+	+	-	-	-	-	-	-	1
13	+	-	+	-	-	-	-	-	1
14	-	+	+	-	-	-	-	-	2

Adapted from Popoff and Vieu (1970) and Popoff and Lallier (1984).

L-arabinose, D-galactose, D-mannose, and D-mannitol. Many strains produce indole. Three subgroups of *A. salmonicida* have been defined based on phenotypic properties (Table 6), and these are sometimes considered as subspecies. A phage typing scheme (Table 7) which may have application in epidemiological studies, has been described by Popoff and Vieu (1970).

## The Genus *Plesiomonas*

*Plesiomonas* is a relatively new genus in the family Vibrionaceae. Since it was first described in 1948, there have been over 250 papers that at least mention it, and several good reviews are available (Brenden et al., 1988; Eddy and Carpenter, 1964; Holmberg and Farmer, 1984; International Workshop, 1986, 1988, 1990; Miller and Koburger, 1985; Sakazaki et al., 1959; Sakazaki and Balows, 1981; Schubert, 1981, 1984; von

Graevenitz, 1980). *P. shigelloides* causes extraintestinal infections of humans and animals, and is occasionally isolated from human feces where at least some strains may cause diarrhea, particularly in the tropics. There is some information on the basic biology of *P. shigelloides*, but it has not been a particularly popular organism for this type of work.

## History, Nomenclature, and Classification of *Plesiomonas*

The organism that is now known as *P. shigelloides* was first described by Ferguson and Henderson (1947), who isolated an organism on MacConkey agar from a fecal specimen and called it "strain C27." They studied the culture with the biochemical and serological methods generally available in 1947 and concluded that strain C27 was "a motile strain of the family

Enterobacteriaceae containing a somatic antigen similar to the major antigen of *Shigella sonnei* phase I..." It was thought to be a member of the "paracolony group" of Enterobacteriaceae.

The classification of Ferguson's C27 group was confusing for many years, and it was classified in a number of different genera and species. Bader (1954) studied a strain of the C27 group and determined that it had a tuft of flagella at one pole similar to the polar flagella of *Pseudomonas*, and proposed the name *Pseudomonas shigelloides*. This was the first proposal of a scientific name, and the species name "shigelloides" has been used for this organism regardless of the genus in which it is classified. Other investigators isolated strains similar to strain C27 and noted the similarity to some of the species in the family Enterobacteriaceae (Cowan, 1956). Cowan (1956) proposed classifying the C27 strains along with *Shigella sonnei* in the genus *Escherichia*. Ewing et al. (1961) studied strains of the C27 group along with a large collection of *Aeromonas* strains and noted that the strains fermented rather than oxidized glucose, and proposed that *Pseudomonas shigelloides* be reclassified in the genus *Aeromonas* as *Aeromonas shigelloides*. Habs and Schubert (1962) studied strains of *Aeromonas shigelloides*, along with strains of *Aeromonas* and other Vibrionaceae, and concluded that *Aeromonas shigelloides* should be removed from the genus *Aeromonas* and classified in a new genus which they named *Plesiomonas*. This proposal agreed with the GC content of the DNA of *Plesiomonas* (51 mol%) compared to *Aeromonas* (57–60 mol%) and *Vibrio* (40–50 mol%). Thus, in 1962, the C27 organism was renamed *Plesiomonas shigelloides*, and Habs and Schubert's classification has become universally accepted with time.

### Classification Based on Nucleic Acid Hybridization and Sequencing

Several studies have used molecular techniques to compare strains of *P. shigelloides* with other species of the Enterobacteriaceae or Vibrionaceae. Fanning et al. (1985) found that strains of *P. shigelloides* were highly related to each other by DNA-DNA hybridization and that *P. shigelloides* was a well defined species based on this criterion. The type strain of *P. shigelloides* was only 8% related by DNA hybridization to *Escherichia coli*, 7% related to the type strain of *Vibrio cholerae* and the 8% related to the type strain of *Aeromonas hydrophila*. *P. shigelloides* was not closely related to any of the other species of Enterobacteriaceae or Vibrionaceae tested. Although *Plesiomonas* has been classified in a number of other genera over the years, including *Aeromonas* (Ewing et al., 1961), *Scatomonas*,

*Fergusonia* (Sebald and Veron, 1963), and *Vibrio* (Hendrie et al., 1971), most authors use the genus name *Plesiomonas* with a single species *P. shigelloides*. However, as described in The Family Vibrionaceae in this Volume, there is a difference of opinion on whether *Plesiomonas* should be classified in the family Enterobacteriaceae or Vibrionaceae. Table 4 in The Family Vibrionaceae in this Volume indicates that *Plesiomonas* has some characteristics in common with both of these families.

MacDonell and Colwell (1985) recently compared the 5S rRNA sequences of a number of Enterobacteriaceae and Vibrionaceae, and concluded that *P. shigelloides* was more closely related to *Proteus mirabilis* and *P. vulgaris* than to any of the species of Vibrionaceae tested. They concluded that *Plesimonas* should be moved to the family Enterobacteriaceae and be included in the genus *Proteus* as *Proteus shigelloides*. The 5S rRNA sequence data of MacDonell and Colwell (1985) also indicated that *Vibrio cholerae* and *V. mimicus* are not closely related, a conclusion difficult to reconcile with the known similarity of these two species, both phenotypically and genetically. This discrepancy must be considered in the evaluation of their proposal.

### Origin of the Name *Plesiomonas*

*Plesiomonas* is derived from the Greek masculine noun "plesios," which means neighbor (to *Aeromonas*), and from the Greek feminine noun "monas" which means "monad" or "unit" (Schubert, 1984). Thus, a literal meaning would be "the neighbor monad," or less literally, "the organism that is neighbor to, or has traditionally been associated with *Aeromonas*." The type species for the genus *Plesiomonas* is *P. shigelloides*, and ATCC 14029 (= NCIB 9242) is the type strain. Many of the scientists that currently study the organism pronounce it *Ple-si-o-mo;pr-nas* (Plee-see-oh-moh;pr-nas), but others pronounce it *Ples;pr-i-o-mo-nas* (Pless;pr-ee-oh-moh-nas). Schubert (Schubert, 1984) gives the former as the pronunciation, which follows the recommendation that has always been given in *Bergey's Manual of Determinative Bacteriology*. The species name "shigelloides" is a modern Latin feminine noun meaning "similar to *Shigella*," or "*Shigella*-like." The species was given this name because many of the original strains agglutinated in *Shigella sonnei* antiserum and were somewhat inactive biochemically like *Shigella*.

### Morphology and Metabolism of *Plesiomonas*

*P. shigelloides* is a small Gram-negative rod measuring 2–3 µm long by 0.7 to 0.8 µm wide (Ewing

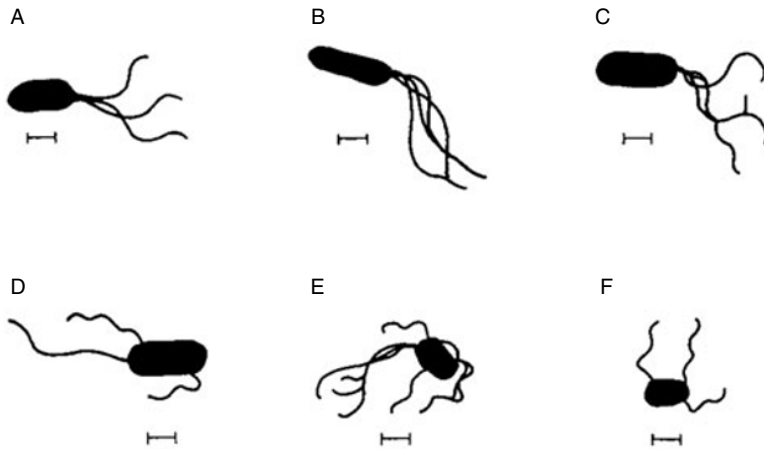


Fig. 2. Cellular morphology of *P. shigelloides*; (A) to (F) are redrawn from photomicrographs of flagellar stains given in Fig. 1 of Ewing et al. (1961); note that both polar and lateral flagella are visible. With flagellar stains, the flagella appear much larger than actual size. Bars = 1  $\mu$ m.

et al., 1961). Most strains are motile and flagellar strains or electron micrographs (Fig. 2) show two to seven flagella located at one end of the cell. Young cultures may also produce lateral flagella with a shorter wave length (Sakazaki and Balows, 1981). There is disagreement among different authors on the length and wave length of the flagella produced (Ewing et al., 1961; Schubert, 1984). Strains of *P. shigelloides* have few other outstanding structural features. Distinct capsules are not produced, but most strains apparently produce a heat-labile capsular substance that blocks agglutination of living cells with homologous O antisera (Shimada and Sakazaki, 1978; 1985). Schubert (1984) stated that *P. shigelloides* does not produce granules, but metachromatic granules that stain with methylene blue have been reported in two studies (Brenden et al., 1988; Pastian, 1984). Brenden et al. (1988) did electron microscopy and showed that structures resembling granules are produced. Their electron micrographs (ruthenium-red staining) also showed a glycocalyx surrounding the cell, which could be the substance that blocks O agglutination. The cellular fatty acid content of *P. shigelloides* strains is similar to that of other species of Enterobacteriaceae and Vibrionaceae (Lambert et al., 1983). Unlike other species of Vibrionaceae, *P. shigelloides* contains the enterobacterial common antigen (Kaluzewski and Czech, 1976; Whang et al., 1972). It also produces lipid A (Basu et al., 1985).

There have been a few studies on the metabolism, physiology, and genetics of *Plesiomonas*. The GC content of DNA is 51 mol% (Sebald and Véron, 1963). Herrington et al. (1987) found that all five strains of *P. shigelloides* contained a single large plasmid that may be important as the genetic determinant of a virulence factor in infections of the intestinal tract. Olsvik et al. (1985) found several different plasmid patterns in a small group of *P. shigelloides* strains isolated

in Peru, and concluded that there was no evidence that *P. shigelloides* produced a plasmid similar to or identical with the *Shigella* invasiveness plasmid. Unlike *Aeromonas*, strains of *P. shigelloides* do not grow at 0–5°C, and only a few strains grow at 8°C (Miller et al., 1986b; Schubert, 1984). The optimum growth temperature for *P. shigelloides* appears to be 38–39°C, with a maximum growth temperature of 40–44°C. About 25% of strains grow at 45°C (Miller et al., 1986b; Schubert, 1984). Strains of *P. shigelloides* may be particularly susceptible to drying. Sakazaki and Balows (1981) reported that unsealed stock cultures in tubes may die after a few days. Strains of *P. shigelloides* grow at pH 5–8 (Schubert, 1984) and may be particularly susceptible to low pH. Janda (1987) found that strains are rapidly killed at a pH below 4 and are also inhibited by substances produced by other intestinal bacteria, such as *Streptococcus faecium* and *Pseudomonas aeruginosa*. These factors may be important in the survival and multiplication of *P. shigelloides* in the human intestinal tract.

## Habitats of *Plesiomonas*

*P. shigelloides* has been isolated from human feces, extraintestinal specimens, animals, and the environment, particularly water (Sakazaki and Balows, 1981; Schubert, 1984), and most strains have been isolated during warm weather. Unlike *Aeromonas*, *P. shigelloides* does not grow at 0–5°C, which may hinder its survival in the environment during the cold months. *P. shigelloides* has been isolated in many different countries.

## Human Extraintestinal Infections

*P. shigelloides* is rarely isolated in most clinical microbiology laboratories but it can occur in a variety of clinical specimens including CSF



(Appelbaum et al., 1978; Dudley et al., 1982; Horst et al., 1982; Su and Choo, 1981), blood (Ellner and McCarthy, 1973; Pathak et al., 1983), wounds, and the respiratory tract. It has also been isolated from cases of cholecystitis, proctitis (Nolte et al., 1988), septic arthritis (Gordon et al., 1983), and endophthalmitis (Cohen et al., 1983). Many literature reports describe cases of meningitis and bacteremia, but this may reflect greater interest in reporting these cases due to their seriousness. *P. shigelloides* causes localized infections after trauma and spontaneous inoculation into the wound of bacteria from an environmental source such as water. A different mechanism involves a gastrointestinal origin from which it is disseminated to other parts of the body. Most patients with serious extraintestinal infections have an underlying disease.

### Human Intestinal Infections

The first reported isolate of *P. shigelloides* (Ferguson and Henderson, 1947) and most subsequent isolates have been from feces. There is a consensus that *P. shigelloides* may be an enteric pathogen in adults and children (McNeeley et al., 1984). A few fatal cases have been reported (Sawle et al., 1986).

However, there is considerable confusion in the literature on the actual etiological role of *P. shigelloides* in particular cases and studies. Many authors have overstated the evidence that *P. shigelloides* actually caused the infection(s). Based on extensive literature reports, we feel it is safe to conclude that "some strain of *P. shigelloides* probably cause diarrhea in at least some people." This would be analogous to the situation with *Escherichia coli* and *Yersinia enterocolitica*, where only certain strains are able to cause diarrhea. The role of the host is also likely to be important.

**HUMANS WITHOUT DIARRHEA** In industrialized countries, *P. shigelloides* is rarely found in the feces of healthy people. The largest culture survey was done in Japan by Arai et al. (1980) who found only 3 carriers among 38,454 (0.0078%) food handlers and school children. A higher carriage rate was found by Pauckova and Fukava (1968). They studied hospitalized patients without diarrhea in Czechoslovakia and found that 15 of 10,643 (0.14%) were positive. In developing countries, the situation is quite different. In Thailand, Pitarangsi et al. (1982) found that 12 of 51 (24%) adults were colonized.

**ANTIBODY RESPONSE AND SERODIAGNOSIS** The results of serological testing for *P. shigelloides* in patients with diarrhea have generally been negative, and these data are viewed as negative

evidence for the etiological role of most *P. shigelloides* strains in diarrhea. Only a few authors have tested stool isolates of *P. shigelloides* with serum to detect antibodies to O or H antigens. Most control sera (patients with no documented exposure to *P. shigelloides*) have no titer or a very low titer (Claesson et al., 1984; Holmberg et al., 1986).

**EPIDEMIOLOGY** *P. shigelloides* is rarely isolated in industrialized countries, but it is frequently found in developing countries with tropical and subtropical climates. Most reports have been about sporadic cases, but a few outbreaks have been reported (Ueda et al., 1963). In developing countries, most diarrhea cases have been exposed to water, animals, or environmental water. Most diarrhea cases that have been positive for *P. shigelloides* have occurred during the summer, which correlates with the frequent presence of the organism in water and the environment during warm weather. In developing tropical countries, drinking water from streams, lakes, and wells is often contaminated with *P. shigelloides* (Bhat et al., 1974; Sanyal et al., 1980; Tsukamoto et al., 1978). *P. shigelloides* strains in water reservoirs can survive purification and occur in tap water.

In industrialized countries, different factors seem to be important in the acquisition of *P. shigelloides*. These include travel to developing countries (Herrington et al., 1987; Holmberg et al., 1986) and eating raw shellfish (Holmberg et al., 1986).

Animals implicated as sources for *P. shigelloides* include oysters (Holmberg et al., 1986; Ratula et al., 1982), fish (Hori et al., 1966), shrimp (Holmberg et al., 1986), and chickens (Newsom and Gallois, 1982).

**TREATMENT** Symptoms in diarrhea patients with feces positive for *P. shigelloides* usually resolve within a few days without specific treatment. Replacement of fluid and electrolytes is important if dehydration is severe (Rolston and Hopfer, 1984). Antibiotic treatment has generally been limited to those with chronic diarrhea or a serious underlying illness. Tetracycline (Reinhardt and George 1985; Rolston and Hopfer, 1984) and trimethoprim-sulfa (Rolston and Hopfer, 1984) usually result in eradication of the organism from the feces and resolution of symptoms. However, it must be emphasized that the etiological role of *P. shigelloides* in most of these reports is uncertain.

### Pathogenesis of Intestinal Infections

Strains of *P. shigelloides* have been isolated from human cases with two different syndromes:

watery diarrhea, suggesting an enterotoxin; and diarrhea with blood and mucus, suggesting an invasive process. There is confusion in the literature whether *P. shigelloides* produces positive results in animal and other models designed to measure or correlate with these two processes. Most authors have studied strains isolated in their particular geographical area, and there have been no comparative studies in different laboratories with the same set of strains.

**HUMAN VOLUNTEER STUDIES** Herrington et al. (1987) fed *P. shigelloides* strain P012 to 33 human volunteers. This strain had been studied by Holmberg et al. (1986) and was from a 4-year-old diarrhea patient whose stool had blood, mucus, and white blood cells; the illness was acquired during foreign travel. None of the volunteers got diarrhea even though some received over  $10^9$  organisms and were given ampicillin to reduce normal intestinal flora. Gut colonization was slight, only 36% even shed the organism for a few days. None of the volunteers had a rise in antibody titer (hemagglutination). These results agreed with the unpublished studies done in Japan (Sakazaki et al., 1959; Sakazaki and Balows, 1981) where a strain of *P. shigelloides* with the 0 antigen of *Shigella sonnei* did not produce diarrhea in human-feeding experiments.

**GERM-FREE PIGLETS** Herrington et al. (1987) obtained some positive results when *P. shigelloides* strains P012 was fed to germ-free piglets. This strain caused mild to moderate diarrhea in 3 of 3 piglets when  $10^9$  cells were given. There was pathology in both the small and large intestine. The organism colonized the small intestine and appeared to penetrate epithelial enterocytes, and could be seen inside vacuoles. Large numbers of organisms were found in the jejunum ( $10^5$ ) and in the distal small intestine ( $10^8$ ). In the large intestine, there was inflammation, mucosal edema, and some disruptions of epithelia. Strain P012 contained a large plasmid which is apparently important, because there were no symptoms or cell penetration when a strain cured of the plasmid was used in the piglet model.

**MONKEYS** Pitarangsi et al. (1982) found negative results when one strain was fed to five Rhesus monkeys, but commented that this might not be a good model since monkeys are often colonized with *P. shigelloides* and may have immunity.

**ADULT RABBITS** Herrington et al. (1987) obtained negative results when their plasmid-containing strain (see above) and several other strains were fed to adult rabbits (intestinal tie, adult rabbit model).

**RABBIT ILEAL LOOPS** There is confusion about whether *P. shigelloides* is positive or negative in the rabbit ileal loop model. Sanyal et al. (1975) reported that all six of their strains were negative for fluid accumulation (live cells injected into intestinal loop segments), but the strains multiplied slightly. Pitarangsi et al. (1982) also found negative results for 27 strains isolated in Thailand. However, positive results have been reported in other studies. Sanyal et al. (1980) found that live cells of 13 strains isolated in India multiplied by  $10^4$  and caused fluid accumulation. Interestingly, several strains required loop-to-loop passage before a positive reaction was obtained. When heated supernatants were injected into loops, there was as much fluid accumulation as when live cells were used. They concluded that a "heat-stable toxin" was responsible for fluid accumulation. Positive results in this model were obtained with live cells by Huq and Islam (1983) and with filtrates by Manorama et al. (1983). The latter authors purified material from the culture filtrates, and concluded that both a heat-labile and a heat-stable enterotoxin might be involved.

**INFANT MOUSE ASSAY** The results in the infant mouse assay are similar to those found with the rabbit ileal loop assay. Several authors found their strains to be uniformly negative (Johnson and Lior, 1981; Pitarangsi et al., 1982), but others obtained positive results (Huq and Islam, 1983; Manorama et al., 1983; Sanyal et al., 1980). Manorama et al. (1983) felt that both a heat-stable and a heat-labile enterotoxin was responsible for fluid accumulation in the infant mouse model, a result similar their results in the rabbit ileal loop.

**TISSUE CULTURE ASSAYS FOR ENTEROTOXINS** Several tissue culture cell lines that traditionally have been used to measure *E. coli* enterotoxins and cholera toxin have been used to test filtrates of *P. shigelloides*. Several authors reported positive results with Y1 cells (mouse adrenal tumor cells) (Gurwith and Williams, 1977; Ljungh et al., 1977; Sanyal et al., 1980) but others reported uniformly negative results (Johnson and Lior, 1981; Pitarangsi et al., 1982). Both Gardner et al. (1987) and Sanyal et al. (1975) obtained positive results for CHO (Chinese hamster ovary) cells, but Johnson and Lior (1981) obtained negative results with a different group of strains. Binns et al. (1984) found some cytopathic effects against HeLa cells. Holmberg et al. (1986) found that their strains isolated in the United States were negative for Shiga-like toxins.

**INVASION OF HeLa CELLS** Binns et al. (1984) found that 3 of 16 strains of *P. shigelloides* were strongly invasive in a manner similar to *Shigella*

strain. Similarly, Olsvik et al. (1985) found that some of their strains isolated in Peru were positive. These results contrast to those of Herrington et al. (1987) who found that all five strains isolated in the United States were negative.

**GUINEA PIG EYE (SERENY TEST)** This model is used to show invasiveness for *E. coli*, *Shigella*, and *Yersinia*. All strains of *P. shigelloides* tested have been negative (Binns et al., 1984; Herrington et al., 1987; Holmberg et al., 1986; Johnson and Lior, 1981; Pitarangsi et al., 1982; Sanyal et al., 1975). In addition, none of the five strains studied by Herrington et al. (1987) reacted with a DNA probe made from the *E. coli-Shigella* invasiveness genes.

**RELATIONSHIPS TO *E. COLI* ENTEROTOXINS** Although few if any strains from the far east have been tested, strains of *P. shigelloides* have been negative when tested in an ELISA assay for *E. coli* heat-labile enterotoxin (Herrington et al., 1987) or by a DNA probe assay for *E. coli* heat-stable toxin (Herrington et al., 1987; Holmberg et al., 1986).

### The Role of *P. shigelloides* in Diarrhea

It is difficult to reconcile some of the conflicting literature reports, but several factors seem important. Different strains have been used by different investigators, so no direct comparisons of results have been made. Most of the studies done in industrialized countries have used strains isolated locally rather than strains isolated in developing countries, which could minimize the likelihood of obtaining pathogenic strains. Different media and growth conditions have been used, which could drastically affect the production of virulence factors, toxins, and other biologically active molecules. Evidence from one study (Sanyal et al., 1980) suggests that strains negative for pathogenicity can become positive after animal passage. This finding, along with the possible role of a virulence plasmid (Herrington et al., 1987), suggests that unstable virulence factors may be involved. For these reasons it seems very desirable to use freshly isolated strains that have not been maintained on laboratory media. In summary, there is some evidence from animal and other virulence models for both enterotoxins and for invasiveness, however, there are no virulence factors that are widely accepted as being important or that should be tested for routinely.

### Animals and *Plesiomonas*

*P. shigelloides* has been isolated from a wide variety of animals, including dogs (Arai et al., 1980), cats (Aldová, 1985; Arai et al., 1980), rats

(Aldová et al., 1980), zoo animals (Bauwens et al., 1983), monkeys (Pitarangsi et al., 1982), chimpanzees (Sakazaki et al., 1959), cattle, pigs (Aldová et al., 1980), poultry, rodents, and shrews (Chenchittikul et al., 1983), vultures (Winsor et al., 1981), turtles (Sugita and Deguchi, 1983), snakes (Davis et al., 1978), fish (Arai et al., 1980; Van Damme and Vandepitte, 1980; Miller and Koburger, 1986a), frogs (Sugita et al., 1985), newts (Arai et al., 1980; Tsukamoto et al., 1978), and shellfish (Arai et al., 1980). The occurrence of *P. shigelloides* in most of these animals is probably related to its frequent occurrence in environmental water.

**ANIMAL DISEASES** Animal diseases due to *P. shigelloides* have rarely been reported. Walsh et al. (1987) reported an infection of the umbilical area (ophthalmitis) and peritonitis in a manatee in Florida. Cruz et al. (1986) reported that *P. shigelloides* causes septicemia in rainbow trout.

### Water

*P. shigelloides* has been isolated frequently from fresh water such as rivers and streams (Arai et al., 1980; Sanyal et al., 1975; Schubert, 1981; Tsukamoto et al., 1978), lakes and ponds (Tsukamoto et al., 1978; Van Damme and Vandepitte, 1980), estuaries, and aquaria for tropical fish (Sanyal et al., 1987). There is considerable variation in the number of organisms present ranging from 10,000 per 100 ml in a Florida estuary to 10 per 100 ml in the river Main in Germany. It appears that *P. shigelloides* does not actually multiply in the water column but may multiply in sediments (Sanyal et al., 1975; Tsukamoto et al., 1978) or in the guts of aquatic animals, who then defecate into the water. Arai et al. (1980) isolated *P. shigelloides* from rivers in Japan, but only in the warmer months (July through November). *P. shigelloides* has also been isolated from well water (Bhat et al., 1974), water treatment plants (Sanyal et al., 1980), and tap water (Tsukamoto et al., 1978). These sources indicate that people can easily be exposed to *P. shigelloides* through drinking water. The organism is probably much more common in the tropics because of the warmer temperatures, and is found more frequently in human feces there because people tend to drink well and surface water. *P. shigelloides* has also been isolated from seawater (Sakazaki and Balows, 1981; Zakhariev, 1971), but it appears to be a transient organism.

### Isolation of *Plesiomonas*

Most laboratories do not use special media or methods to isolate *P. shigelloides*. Thus, if this

species is greatly outnumbered by other organisms it will probably be missed. This led to the development and evaluation of special media and methods.

### Normal Plating Media

*P. shigelloides* grows well on most plating media used in enteric bacteriology and forms colonies 1–2 mm in diameter after 24 hours of incubation. On nutrient agar *P. shigelloides* forms moist smooth colonies (colony type 1), but many strains contain up to four other colony types (Sakazaki et al., 1959). Colony type 1 was considered to be the parent colony type, which dissociated into the other types. On blood agar *P. shigelloides* forms smooth colonies without hemolysis, although a few hemolytic strains have been reported (Aldová et al., 1966; Zajc-Satler et al., 1972). Most strains of *P. shigelloides* are colorless or pink on MacConkey agar, but a few strains ferment lactose rapidly and are red. Old stock cultures of these strong-lactose-fermenting strains will usually have white, pink, and red colonies on MacConkey agar, and thus appear to be a mixed culture. Strains of *P. shigelloides* also grow on deoxycholate agar and Hektoen agar, and usually appear as nonlactose fermenters. In a study of *P. shigelloides* from fresh fish in Zaire, Van Damme and Vandepitte (1980) found that MacConkey agar was a better plating medium than XLD or SS agar for plating subcultures from tetrathionate broth enrichments.

### Special Media for *Plesiomonas*

Several media and methods have been designed to selectively isolate *P. shigelloides*. Although *P. shigelloides* is moderately resistant to ampicillin, the ampicillin-containing selective media designed for the isolation of *Aeromonas* are too inhibitory and have not proved useful in isolation. Brilliant green has been used in several broths and agars. Strains of *P. shigelloides* grow in the presence of 0.0005 g/l brilliant green (Millership and Chattopadhyay, 1984; Schubert, 1977) per l but 0.01 g/l is inhibitory (Millership and Chattopadhyay, 1984). Strains of *P. shigelloides* are also resistant to bile salts, which are usually incorporated in media to inhibit Gram-positive bacteria. Most bacterial species do not ferment myo-inositol, but almost all strains of *P. shigelloides* ferment this naturally occurring cyclic polyhydroxyl alcohol. Schubert (1977) took advantage of the three properties discussed above, and designed inositol-brilliant green-bile salts agar.

### Inositol-Brilliant Green-Bile Salts Agar (*Plesiomonas* Differential Agar)

The following formula is the one given by given by E. Merck, Germany, which at one time produced and sold this medium under the name “*Plesiomonas* Differential Agar,” catalog no 15106.

Peptone	7.5 g
Meat extract	7.5 g
Sodium chloride	5 g
Bile salts mixture	8.5 g
Brilliant green	0.00033 g
Neutral red	0.025 g
Inositol	10 g
Agar	13.5 g
Water	1 liter

Agar pH 7.2. Suspend the ingredients in the water and heat to boiling to dissolve. Autoclave at 121°C for 15 min. Cool to 45°C and pour into petri dishes.

Inoculate the plates and incubate at 37°C for 48 h. Colonies of *P. shigelloides* ferment the inositol and appear red. Merck states that “whitish *P. shigelloides* colonies grow after incubating for a maximum of 48 hours; they display a reddish colouration the intensity of which depends on the distance separating them from neighboring colonies.”

Miller and Koburger (1986a) found that inositol-brilliant green-bile salts agar was more effective than *Plesiomonas* agar, and that strains grew poorly if they had been stressed by heat or cold. This suggests that a period of pre-enrichment in a nonselective broth may be desirable before the use of more inhibitory media. Huq and Akhter (International Workshop, 1988) compared modified Salmonella-Shigella agar, inositol-brilliant green-bile salts agar, and *Plesiomonas* differential agar, and found the best results with *Plesiomonas* differential agar incubated at 42°C for 24 h, rather than at 37°C for 48 h.

## Identification of *Plesiomonas*

*P. shigelloides* is easy to identify with standard biochemical tests or commercial identification systems. *P. shigelloides* is oxidase positive, which differentiates it from species of Enterobacteriaceae with which it could otherwise be confused. Strains of *P. shigelloides* ferment myo-inositol; are positive for lysine decarboxylase, ornithine decarboxylase, and arginine dihydrolase; and do not require added sodium chloride for growth. This unique pattern differentiates it from other species of Vibrionaceae.

### Biochemical Characteristics

Table 8 gives the biochemical characteristics of strains that were studied in our laboratory and compares the results with the 280 strains studied by Sakazaki and Balows (1981) and with the data from other reference laboratories (Holmes et al., 1986). Most of the positive biochemical reactions

Table 8. Biochemical reactions and other properties of *Plesiomonas shigelloides* as reported by different authors.

Test or property	Composite result <sup>b</sup>	Results obtained by:			
		Our laboratory; 70 strains <sup>c</sup>	Sakazaki and Balows (1981); 280 strains	Ewing et al. (1961); 50 strains	Holmes et al. (1986)
Indole production	99 <sup>e</sup>	100	100	98 (100)	100
Methyl red	97	88	100	100	100
Voges-Proskauer (O'Meara)	0	0	0	0	0
Citrate utilization (Simmons')	0	0	0	0	0
H <sub>2</sub> S on triple sugar iron agar	0	0	0	0	0
H <sub>2</sub> S, paper method	17				17
Urea hydrolysis (Christensen)	0	0	0	0	0
Phenylalanine deaminase	5 <sup>d</sup>	3 <sup>d</sup>	0	44 <sup>d</sup>	0
Lysine decarboxylase (Moeller)	99	100	99.3 (100)	98	100
Arginine dihydrolase (Moeller)	98 (99)	98.5 (100)	97.8 (99.5)	94 (98)	100
Ornithine decarboxylase (Moeller)	93	100	91.4 (94.2)	48	100
Motility	90	94		86	83
Gelatin hydrolysis (22°C)	0	0	0	0	0
KCN test (% resistant to cyanide)	1	0	1.8	0	0
Malonate utilization	0	0	0	0	0
D-Glucose: acid production	100	100	100	100	100
D-Glucose: gas production	0	0	0	0	0
Acid production from:					
Adonitol	0	0	0	0	0
L-Arabinose	0	0	0	0	0
D-Arabitol	0	0			
Cellobiose	0	0	0	0	0
Dulcitol	0	0	0	0	0
Erythritol	0	0	0		
D-Galactose	79	79			
Glycerol	50	38 (80)		12	67
myo-Inositol	98	97	98.2 (100)	100	100
Lactose	70	82 (85)	40 (92.8)	66 (92)	89
Maltose	90	100	62.8	54	99
D-Mannitol	0	0	0	0	0
D-Mannose	11 (26)	11 (26)			
Melibiose	60	67 (78)	40 (50)		
α-Methyl-D-glucoside	0	0			
Raffinose	0	0	0	0	0
L-Rhamnose	0	0	0	0	0
Salicin	5	0	11.7 (22)	34	11
D-Sorbitol	0	0	0	0	0
Sucrose	0 (2)	0	0 (2.8)	0 (6)	0
Trehalose	100	100	100	96 (98)	100
D-Xylose	0	0	0	0	0
Esculin hydrolysis	0	0	0		
Mucate fermentation	0	0	0	0	0
Tartrate fermentation (Jordan)	50	50			88
Acetate utilization	6 (21)	6 (21)			
Lipase (corn oil)	0	0			0
Lipase (Tween-80)	0		0		
DNase (25°C)	0 (7)	0 (7)	0		0
Nitrate reduction to nitrite	100	100	100	100	100
Oxidase (Kovacs)	99	97	100	100	100
Catalase	100		100	100	100
ONPG test	90	82	100		89
Yellow pigment production	0	0			0
Citrate utilization (Christensen)	0	0		0	0
Tyrosine clearing	0	0			
Hemolysis on sheep blood	2				
Glucose as sole carbon source	72			72 (90)	
MacConkey agar, growth on	100				100
Gluconate	0				0
Casein hydrolysis	0				0

<sup>a</sup>The numbers give the percentage positive (after 48 h of incubation for our laboratory's results). Delayed reactions are given in parentheses (cumulative percentage positive at 7 days for our laboratory's data).

<sup>b</sup>These are arbitrary values based on examination of all data available; in most cases, they are weighted more heavily toward our results.

<sup>c</sup>Thirty-six strains were studied with a complete set of biochemical reactions; 34 were studied with fewer tests.

occurred at 24 hours, but some were delayed. For most tests, there is little strain-to-strain variation, which indicates there are few true biotypes of *P. shigelloides*. The variation in lactose fermentation is important because this sugar is an ingredient in many enteric plating media.

**COMMERCIAL IDENTIFICATION SYSTEMS** Several studies have indicated that the API 20E system accurately identifies *P. shigelloides* (Overman et al., 1985; Penn et al., 1982; Rolston and Hopfer, 1984; von Graevenitz and Bucher, 1983). The time of incubation could perhaps be reduced to 5 hours and still give a correct identification (Overman

and Overley, 1986). Other commercial systems have been studied less frequently, but several apparently give good results. These include the Enterotube (Holmes et al., 1977), Abbott Advantage—5 hr (Jorgensen et al., 1984), Pathotec system (Holmes et al., 1977), and Quantum II system (Sylvester and Washington, 1984). Most of the commercial identification systems have now include *P. shigelloides* in their data bases.

### Serotyping

At least three different serotyping systems have been described for *Plesiomonas*. Sakazaki et al.

Table 9. The antigenic schema for *P. shigelloides* described by Shimada and Sakazaki.

Serotype (O and H antigen)	Number of strains	Serological cross-reactions
O1:H1a,1b	1	
O2:H1a,1c	2	
O3:H2	8	
O4:H3	1	
O5:H4	5	
O6:H3	1	
O7:H2	1	
O7:H- (nonmotile)	1	
O8:H3	1	
O8:H5	4	
O9:H2	1	
O10:H11	1	
O11:H2	3	O11 is related to <i>Shigella dysenteriae</i> O8
O11:H5	1	
O12:H2	1	
O12:H3	1	
O12:H9	2	
O13:H2	2	
O14:H4	1	
O14:H5	2	
O15:H10	1	
O16:H5	2	
O17:H2	12	O17 is identical to <i>Shigella sonnei</i>
O17:H6	1	
O18:H2	1	
O19:H2	1	
O20:H2	2	
O21:H7	1	
O21:H8	1	
O22:H3	8	O22 is related to <i>Shigella dysenteriae</i> O7
O22:H5	1	
O22:H8	3	
O23:H1a,1c	1	O23 is related to <i>Shigella boydii</i> O13
O24:H5	3	
O24:H8	2	
O25:H3	1	
O26:H1a,1c	1	
O27:H3	1	
O28:H3	1	
O29:H2	1	
O30:H1a,1c	1	
TOTAL	87	

Adapted from Sakazaki and Balows (1981).

(1959) studied Japanese strains and defined 50 O and 4 H antigens. Over the years, this schema has been expanded (Shimada and Sakazaki 1978), and now contains 50 O and 17 H antigens (Shimada and Sakazaki, 1985). Other serotyping schemas include those of Quincke (1967) with 16 O antigens, and Aldová and Geizer (1968) with 13 O and 5 H antigens, which has now been expanded to 30 O antigens (Aldová, 1985). Serotyping has not proved as useful as expected in defining the role of *P. shigelloides* in causing diarrhea, but it was used in several studies to show that human cases and water isolates had the same O and H antigens (Tsukamoto et al., 1978).

**RELATIONSHIP TO *SHIGELLA SONNEI*** *P. shigelloides* was originally discovered because it agglutinated in *Shigella sonnei* antiserum (Ferguson and Henderson, 1947). Other authors have noted this cross-reaction, but only about 25% of *P. shigelloides* strains have this serological relationship (Holmberg et al., 1986; Pitarangsi et al., 1982; Shimada and Sakazaki, 1985; Vandepitte et al., 1957). Only the strains of *P. shigelloides* that contain O antigen 17 (Table 9) agglutinate in the *S. sonnei* serum. Sack (International Workshop, 1988) speculated that the identity of the *Shigella sonnei* O antigen with *P. shigelloides* O antigen 17 may explain the rarity of *Shigella sonnei* in developing countries. In these countries, environmental exposure to *P. shigelloides* O 17 antigen would provide immunological protection against infection with *S. sonnei*. Because of better hygiene in industrialized countries, there would be less exposure to environmental strains of *P. shigelloides* and thus no protection against infection with *S. sonnei*.

### Antibiotic Susceptibility

*P. shigelloides* is generally susceptible to antibiotics including many newly introduced ones (Dahm and Weinberg, 1980; Holmberg et al., 1986; Miller et al., 1983; O'Hare et al., 1985; Rolston and Hopfer, 1984), such as imipenem, ceftazidime, cefotiam, ceftriaxone, and cefotaxime (Brenden et al., 1988), and to the new quinolone antibiotics (O'Hare et al., 1985). Reports of antibiotic resistance have been rare. Holmberg et al. (1986) found occasional resistance to the sulfa drugs and to tetracycline. However, Olsvik et al. (1985) found that many of their strains isolated in Peru were resistant to gentamicin. There is some confusion in the literature about the resistance of *P. shigelloides* to ampicillin and other penicillins.

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- International Workshop. 1986. The First International Workshop on *Aeromonas* and *Plesiomonas* was held in Manchester England on 5–6 September 1986, as a satellite meeting of the XIV International Congress of Microbiology. One to three page summaries of the talks and posters sessions were published in an 87 page printed booklet. The journal *Experientia* (volume 43, pages 348–374, 1987) published summaries of 20 invited talks, but not the posters.
- International Workshop. 1988. The Second International Workshop on *Aeromonas* and *Plesiomonas* was held at the Eden Rock Hotel in Miami, Florida on 7 May 1988, in conjunction with the American Society for Microbiology's annual meeting. One to two page summaries of 13 talks and 1/2 page abstracts from the 39 posters were published in a 72 page printed booklet. (Copies were originally available and may still be from Dr. R. R. Colwell or Dr. S. W. Joseph, Department of Microbiology University of Maryland, College Park, Maryland 20742).
- International Workshop. 1990. The Third International Workshop on *Aeromonas* and *Plesiomonas* was held in LO-Skolen in Helsingör (Elsinore) Denmark on 5–6 September 1990. Details are available from Torkel Wadström and Åsa Ljungh, University of Lund, Department of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.
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# The Genus *Alteromonas* and Related Proteobacteria

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## Introduction

Many marine heterotrophic Gram-negative aerobic or facultatively anaerobic bacteria are affiliated to the genera *Alteromonas*, *Pseudoalteromonas*, *Glaciecola*, *Idiomarina* and *Colwellia*. They belong to the  $\gamma$ -subclass of the class *Proteobacteria* (Stackebrandt et al., 1988) and share similar phenotypic, genotypic and phylogenetic characteristics. These bacteria, essential components of the marine part of the biosphere, have very diverse habitats including coastal and open water areas, deep-sea and hydrothermal vents, and marine sediments. The genus *Alteromonas* was created by Baumann et al. (1972) for marine Gram-negative heterotrophic bacteria motile by a single polar flagellum, with an oxidative metabolism and a DNA G+C content of 38–50 mol%. On the basis of 16S rDNA gene sequence analysis, the genus *Alteromonas* was revised in 1995 to contain only one species, *A. macleodii*. The remaining species were reclassified as *Pseudoalteromonas* species (Gauthier et al., 1995). The genus *Glaciecola* was proposed by Bowman et al. (1998c) for two groups of psychrophilic, strictly aerobic chemoheterotrophic bacteria isolated from sea-ice diatom assemblages from coastal area of eastern Antarctica. Two deep-sea bacteria were isolated from seawater of the Pacific Ocean and described as species of the new genus *Idiomarina* (Ivanova et al., 2000b). Deming and coauthors accommodated facultatively anaerobic, psychrophilic and barophilic bacteria into the genus *Colwellia* (Deming et al., 1988). Recently a new genus, *Thalassomonas*, comprising marine pigmented bacteria that are phylogenetic neighbors of *Colwellia* was described by Macian et al. (2001).

## History of the *Alteromonas* and *Pseudoalteromonas* Genera

Through 1994

In the 1950s, marine aerobic heterotrophic Gram-negative and nonfermentative *Pseudomonas*-like

bacteria with the low DNA G+C content of 40–50 mol% were assigned to the genus *Pseudomonas*, including *P. atlantica*, *P. carrageenovora* (Humm, 1946; Yaphe, 1955; Yaphe, 1957), *P. putrefaciens* (Lee et al., 1981) and *P. piscicida* (Bein, 1954; Buck et al., 1963). The numerical taxonomy and some identification schemes of marine *Pseudomonas*-like bacteria have been considered in a number of studies (Shewan et al., 1960; Gibson et al., 1977; Lee et al., 1977; Gray and Stuart, 1980; Oliver, 1982).

In 1972, Baumann and coauthors characterized 218 marine bacterial strains isolated from seawater samples collected in the area of the Hawaiian archipelago. Most of these bacteria were aerobic, Gram-negative and heterotrophic microorganisms, motile by a single polar flagellum. Bacteria with a DNA G+C content between 57.5 and 64.7 mol% were identified as members of the genus *Pseudomonas*. The remaining strains with a lower DNA G+C content (between 43 and 48 mol%) were affiliated to the genus *Alteromonas*. Four phenotypically distinguishable clusters were described as new species, i.e., *A. communis*, *A. vaga*, *A. macleodii* and *A. marinopraesens* (Baumann et al., 1972). *Alteromonas marinopraesens* was later renamed *A. haloplanktis* (Reichelt and Baumann, 1973).

DNA-rRNA and DNA-DNA hybridization experiments among *Alteromonas* species revealed the genetic heterogeneity of the genus *Alteromonas* and identified four distinct clusters: 1) *Alteromonas macleodii*; 2) the *Alteromonas haloplanktis* cluster, including the majority of *Alteromonas* species and some undescribed taxa, i.e., *A. haloplanktis*, *A. espejiana*, *A. undina*, *A. rubra*, *A. luteoviolacea*, *A. citrea*, *A. aurantia*, and also “*Pseudomonas piscicida*,” “*Alteromonas thalassomethanolica*,” “*Pseudomonas marinoglutinosa*,” “*Pseudomonas nigrifaciens*,” “*Pseudomonas carrageenovora*,” “*Pseudomonas atlantica*” and a number of unidentified strains named “alginolytic bacteria”; 3) *A. putrefaciens* and *A. hanedai*; 4) *A. communis* and *A. vaga*, which formed a separate rRNA branch, and were excluded from *Alteromonas* to create a new genus of marine bacteria *Marinomonas* (Van Landschoot and DeLey, 1983). The latter group

differs from the first three groups in the inability to produce extracellular hydrolases and utilize glycerol, lactate, aromatic compounds and other carbon sources. Preliminary studies on the amino acid sequence composition of glutamine synthetase (Baumann and Baumann, 1980) and nucleotide divergence of ribosomal RNA (De Smedt et al., 1980) of *Alteromonas* species confirmed the distinct position of *A. communis* and *A. vaga*.

Pigmented heterotrophic *Alteromonas*-like bacteria have been isolated from the Mediterranean Sea coastal waters. Some of them produced the pigment violacein and revealed antibiotic activity. As the new violet-pigmented isolates differed from the violacein-containing *Chromobacterium* species in physiological and genetic properties, they were described as a new species, *Alteromonas luteoviolacea* (Gauthier, 1976b; Gauthier, 1982). The other antibiotic-producing isolates, characterized by the formation of different intracellular indiffusible red, orange and yellow pigments, were described as three novel *Alteromonas* species, i.e., *A. rubra*, *A. aurantia* and *A. citrea*, respectively (Gauthier, 1976a; Gauthier, 1977; Gauthier and Breittmayer, 1979).

Enger et al. (1987) investigated twelve aerobic Gram-negative prodigiosin-producing marine bacteria with a DNA G+C content of 37 mol%. The strains differed from known *Alteromonas* species in the ability to denitrify and in the possession of sheathed flagella. Despite their distinctive properties, all new isolates were included in the new species, *Alteromonas denitrificans* (Enger et al., 1987). It should be noted that cells with sheathed flagella were observed earlier in strains of *Pseudoalteromonas luteoviolacea* (Novic and Tyler, 1985).

Nonpigmented bacteria were described as *A. espejiana* and *A. undina* (Chan et al., 1978) and *A. hanedai* (Jensen et al., 1980). It is pertinent to note that *A. espejiana* was studied initially intensively because it was the host of an unusual lipid containing bacteriophage PM2 (Espejo and Canelo, 1968). *Alteromonas espejiana* produces the exonuclease BAL-31, phospholipase A, and lysophospholipase (Cadman and Eichberg, 1983; Wei et al., 1983).

Several pseudomonads with a low DNA mol% G+C content, including *P. atlantica*, *P. putrefaciens*, *P. piscicida* and *P. nigrifaciens* were included into *Alteromonas* as species *incertae sedis* (Baumann et al., 1984b). Yellow-pigmented bacteria causative of fish disease were originally identified as *Flavobacterium piscicida* (Bein, 1954), and described as *Pseudomonas piscicida* (Buck et al., 1963) when similar bacteria were later detected in seawater during the "red tide" of the microalgae *Gymnodium brevis*.

*Pseudomonas nigrifaciens* described earlier by White (1940) was reclassified as *A. nigrifaciens* (Baumann et al., 1984a). On the basis of 5S rRNA sequence analysis, *A. putrefaciens* (Lee et al., 1981) and *A. hanedai* (Jensen et al., 1980) were reclassified and assigned to the new genus *Shewanella* (MacDonell and Colwell, 1985).

DNA-rRNA hybridization were used to study the genotypic relationships between species of *Alteromonas*, *Pseudomonas* and other *Pseudomonas*-like bacteria. As a result of these experiments and supported by morphological, physiological, and biochemical properties and by DNA base composition, 11 strains of five *Pseudomonas* species (*P. atlantica*, *P. carrageenovora*, *P. marinoglutinosa*, *P. nigrifaciens* and *P. piscicida*) were assigned to the genus *Alteromonas* (De Vos et al., 1989). Though "*Pseudomonas atlantica*" and "*Pseudomonas carrageenovora*" were included in *Alteromonas*, their species status was not elucidated. Later, several aerobic Gram-negative polarly flagellated agar-decomposing bacteria were isolated from marine seaweeds collected in coastal waters of the Sea of Japan. On the basis of the phenotypic properties and DNA-DNA hybridization results of five newly isolated strains, *Pseudomonas atlantica* and *Pseudomonas carrageenovora* (capable of agar or carrageenan hydrolysis) were described as the new species *Alteromonas atlantica* and *Alteromonas carrageenovora*, respectively (Akagawa-Matsushita et al., 1992b).

The tetrodotoxin-synthesizing bacteria of *A. tetraodonis* were isolated from skin slime of the fish *Fugu poecilonotus*—a well-known producer of tetrodotoxin (Simidu et al., 1990). DNA-DNA hybridization experiments led to the reclassification of *A. tetraodonis* as *A. haloplanktis* subsp. *tetraodonis* and *A. haloplanktis* as *A. haloplanktis* subsp. *haloplanktis* (Akagawa-Matsushita et al., 1993), which were later included with *Pseudoalteromonas* (Gauthier et al., 1995). However, on the basis of the polyphasic taxonomic study, *P. haloplanktis* subsp. *tetraodonis* strain (originally described as *A. tetraodonis* by Simidu et al., 1990) was revived as an independent species *Pseudoalteromonas tetraodonis*, and *P. haloplanktis* subsp. *haloplanktis* was reclassified as *Pseudoalteromonas haloplanktis* (Ivanova et al., 2001c).

## 1995 to the Present

The fundamental systematic investigation of marine aerobic proteobacteria was published by G. Gauthier and coauthors (Gauthier et al., 1995). To characterize the intra- and intergeneric relationships of *Alteromonas* and related genera, small-subunit (16S) ribosomal RNA sequence



analysis was performed on 17 strains of *Alteromonas*, *Shewanella*, *Vibrio* and *Pseudomonas* species using three different phylogenetic algorithms. All species belong to the  $\gamma$ -3 subgroup of the class Proteobacteria. The molecular analysis confirmed the genetic heterogeneity of the genus *Alteromonas* and demonstrated the separate phylogenetic position of *Alteromonas macleodii* from the other species forming a monophyletic cluster. The new genus *Pseudoalteromonas* was proposed to include the remaining 11 earlier described *Alteromonas* species and *Pseudomonas piscicida*. On the basis of the phylogenetic study of Gauthier et al. (1995), *Pseudoalteromonas haloplanktis* ATCC 14393<sup>T</sup> was described as a type strain and type species of the new genus. In addition, the following species were reclassified: *Pseudoalteromonas atlantica*, *Pseudoalteromonas aurantia*, *Pseudoalteromonas carrageenovora*, *Pseudoalteromonas citrea*, *Pseudoalteromonas denitrificans*, *Pseudoalteromonas espejiana*, *Pseudoalteromonas luteoviolacea*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas piscicida*, *Pseudoalteromonas rubra* and *Pseudoalteromonas undina*. The study pointed towards the presence of four monophyletic groups that were confirmed by different phylogenetic algorithms: 1) *Pseudoalteromonas denitrificans* as an outgroup species; 2) two pigmented species, *Pseudoalteromonas citrea* and *Pseudoalteromonas aurantia*; 3) three other pigmented species, *Pseudoalteromonas piscicida*, *Pseudoalteromonas rubra* and *Pseudoalteromonas luteoviolacea*; and 4) all nonpigmented *Pseudoalteromonas* species (Gauthier et al., 1995). Thus, the history of the two genera *Alteromonas* and *Pseudoalteromonas* is very closely interwoven (Table 1).

*Alteromonas fuliginea* and *A. distincta* (Romanenko et al., 1994b; Romanenko et al., 1995a) have been described as pigmented melanin-synthesizing *Alteromonas* species and were reclassified as *Pseudoalteromonas* species (Ivanova et al., 1998b; Ivanova et al., 2000a; Table 1).

The new species of encapsulated bacteria of *Alteromonas*, *A. elyakovii*, was described for a strain isolated from the mussel *Crenomytilus grayanus* inhabitant of the Peter the Great Bay, Sea of Japan (Ivanova et al., 1996b). *Alteromonas elyakovii* possessed highly active alkaline phosphatase and laminaranase (Fedosov et al., 1991; Sova et al., 1994). Bacteria with similar phenotypic and genotypic characteristics were isolated from spot-wounded fronds of *Laminaria japonica*, collected from the Sea of Japan (Sawabe et al., 1992). The *Laminaria* isolates were characterized by unique alginolytic activity and might be the causative agent of spot disease (Sawabe et al., 1997; Sawabe et al., 1998a). Phy-

logenetic investigations of strain *Alteromonas elyakovii* KMM 162 and the alginolytic strains showed that they belong to the same species *Pseudoalteromonas elyakovii* (Sawabe et al., 2000).

Bacteria associated with mollusks were described as the first marine isolates of *P. nigrifaciens* and the species description was emended (Ivanova et al., 1996a).

During the last years, the genus *Pseudoalteromonas* was enlarged with the new species *P. antarctica* (Bozal et al., 1997), *P. prydzensis* (Bowman, 1998a), *P. bacteriolytica* (Sawabe et al., 1998a), *P. tunicata* (Holmstrom et al., 1998), *P. peptidolytica* (Venkateswaran and Dohmoto, 2000) and *P. ulvae* (Egan et al., 2001).

Besides the description of the new species, the literature indicates the presence of additional species, which, however, have not been validly described. Andrykovitch and Marx (1988) reported on the isolation of the new melanin-synthesizing bacterium “2-40” associated with the alga *Spartina alterniflora*, which is able to hydrolyze agar and other polysaccharides. The tyrosinase from strain 2-40 has been characterized (Kelley et al., 1990). According to its physiological and biochemical properties and its DNA G+C content of 45.6 mol%, strain 2-40 was assigned to the genus *Alteromonas*, but not identified at the species level. Analysis of the 16S ribosomal DNA sequence demonstrated that the bacterium probably represents the type of a new hitherto undescribed genus (Gonzalez and Weiner, 2000).

In 1993, the yellow-gray pigmented “*Alteromonas rava*” sp. nov. [inv.] synthesizing the new antibiotics “thiomarinols” was described (Kodama et al., 1993; Shiozawa et al., 1993; Shiozawa and Takahashi, 1994), but currently the species has not yet been validated.

A mesophilic heterotrophic bacterium, isolated from a seawater sample collected near a deep-sea hydrothermal vent, was identified as *Alteromonas macleodii* according to its morphological, physiological, biochemical and genotypic properties (Raguene et al., 1996). Analysis of 16S rDNA confirmed the phylogenetic similarity, which was further substantiated by a DNA-DNA reassociation of 82%. The authors classified the new bacterium as *A. macleodii* subsp. *fijiensis* on the basis of the following findings: DNA-DNA relatedness level lower than 90% but higher than 70%; metabolic differences between the type and the new strain; the ability of the new bacterium to produce acidic exopolysaccharide with novel properties; and the isolation source of the new bacterium, which is a deep-sea hydrothermal vent, depth 2000 m (Raguene et al., 1996). However, the subspecies name *fijiensis* has yet not been validated.

Table 1. List of *Alteromonas* bacteria reclassified as valid species of *Pseudoalteromonas* or other genera.

Description	Author	Reclassified as	References
<i>Alteromonas communis</i>	Baumann et al., 1972	<i>Marinomonas communis</i>	Van Landschoot and DeLey, 1983
<i>Alteromonas vaga</i>	Baumann et al., 1972	<i>Marinomonas vaga</i>	Van Landschoot and DeLey, 1983
<i>Alteromonas macleodii</i>	Baumann et al., 1972	Emended <i>Alteromonas macleodii</i>	Gauthier et al., 1995
<i>Alteromonas marinopraesens</i> (including former <i>Vibrio haloplanktis</i> ) renamed <i>Alteromonas haloplanktis</i>	Baumann et al., 1972 Zobell and Upham, 1944 Reichelt and Baumann, 1973	<i>Alteromonas haloplanktis</i> subsp. <i>haloplanktis</i>	Akagawa-Matsushita et al., 1993
<i>Alteromonas haloplanktis</i> subsp. <i>haloplanktis</i>	Akagawa-Matsushita et al., 1993	<i>Pseudoalteromonas haloplanktis</i> subsp. <i>haloplanktis</i>	Gauthier et al., 1995
<i>Pseudoalteromonas haloplanktis</i> subsp. <i>haloplanktis</i>	Gauthier et al., 1995	Emended <i>Pseudoalteromonas haloplanktis</i> subsp. <i>haloplanktis</i>	Ivanova et al., 2001c
<i>Alteromonas tetraodonis</i>	Simidu et al., 1990	<i>Alteromonas haloplanktis</i> subsp. <i>tetraodonis</i>	Akagawa-Matsushita et al., 1993
<i>Alteromonas haloplanktis</i> subsp. <i>tetraodonis</i>	Akagawa-Matsushita et al., 1993	<i>Pseudoalteromonas haloplanktis</i> subsp. <i>tetraodonis</i>	Gauthier et al., 1995
<i>Pseudoalteromonas haloplanktis</i> subsp. <i>tetraodonis</i>	Gauthier et al., 1995	<i>Pseudoalteromonas tetraodonis</i>	Ivanova et al., 2001c
<i>Alteromonas luteoviolacea</i>	Gauthier, 1976b, 1982	<i>Pseudoalteromonas luteoviolacea</i>	Gauthier et al., 1995
<i>Alteromonas rubra</i>	Gauthier, 1976a	<i>Pseudoalteromonas rubra</i>	Gauthier et al., 1995
<i>Alteromonas aurantia</i>	Gauthier, 1977	<i>Pseudoalteromonas aurantia</i>	Gauthier et al., 1995
<i>Alteromonas citrea</i>	Gauthier and Breittmayer, 1979	<i>Pseudoalteromonas citrea</i>	Gauthier et al., 1995
<i>Alteromonas fuliginea</i>	Romanenko et al., 1994b	<i>Pseudoalteromonas citrea</i>	Ivanova et al., 1998b
<i>Alteromonas denitrificans</i>	Enger et al., 1987	<i>Pseudoalteromonas denitrificans</i>	Gauthier et al., 1995
<i>Alteromonas espejiana</i>	Chan et al., 1978	<i>Pseudoalteromonas espejiana</i>	Gauthier et al., 1995
<i>Alteromonas undina</i>	Chan et al., 1978	<i>Pseudoalteromonas undina</i>	Gauthier et al., 1995
<i>Pseudomonas piscicida</i> (former “ <i>Flavobacterium piscicida</i> ”)	Buck et al., 1963 Bein, 1954	<i>Pseudoalteromonas piscicida</i>	Gauthier et al., 1995
<i>Alteromonas nigrifaciens</i> (former “ <i>Pseudomonas nigrifaciens</i> ”)	Baumann et al., 1984a White, 1940	<i>Pseudoalteromonas nigrifaciens</i> Emended <i>Pseudoalteromonas nigrifaciens</i>	Gauthier et al., 1995 Ivanova et al., 1996a
<i>Alteromonas atlantica</i> (including former “ <i>Pseudomonas atlantica</i> ”)	Akagawa-Matsushita et al., 1992b Humm, 1946 Yaphe, 1955, 1957	<i>Pseudoalteromonas atlantica</i>	Gauthier et al., 1995
<i>Alteromonas carrageenovora</i> (former “ <i>Pseudomonas carrageenovora</i> ”)	Akagawa-Matsushita et al., 1992b Yaphe, 1955	<i>Pseudoalteromonas carrageenovora</i>	Gauthier et al., 1995
<i>Alteromonas distincta</i>	Romanenko et al., 1995a	<i>Pseudoalteromonas distincta</i>	Ivanova et al., 2000a
<i>Alteromonas elyakovii</i>	Ivanova et al., 1996b	<i>Pseudoalteromonas elyakovii</i>	Sawabe et al., 2000
<i>Alteromonas putrefaciens</i>	Lee et al., 1981	<i>Shewanella putrefaciens</i>	MacDonell and Colwell, 1985
<i>Alteromonas hanedai</i>	Jensen et al., 1980	<i>Shewanella hanedai</i>	MacDonell and Colwell, 1985
<i>Alteromonas colwelliana</i> “ <i>Alteromonas thalassomethanolica</i> ”	Weiner et al., 1988 Yamamoto et al., 1980	<i>Shewanella colwelliana</i> <i>Methylophaga thalassica</i>	Coyne et al., 1989 Janvier et al., 1985

Raguene et al. (1997) proposed a new *Alteromonas* species, *Alteromonas infernus*, for a deep-sea polysaccharide-producing bacterium. The new strain was isolated from a sample of fluid collected from the surface of the vestimentiferan worm *Riftia pachyptila*, which inhabits sites near hydrothermal vents. Morphological and physiological properties of these bacteria

were similar to those of *Alteromonas macleodii*, e.g., rod-shaped cells motile by means of a single polar flagellum. The DNA G+C content was 48 mol%. The level of the DNA-DNA reassociation between the new bacterium and *A. macleodii* was 48%, indicating that the new isolate may indeed constitute a new species of *Alteromonas* (Raguene et al., 1997). *Alteromonas infernus*,

however, has not yet been included in the Validation Lists.

## Genus *Alteromonas*

### Phylogeny

The results of phylogenetic analysis, based on 16S rDNA sequence, revealed *Alteromonas macleodii* belongs to the  $\gamma$ -3 subgroup of the class Proteobacteria. It forms a monophyletic taxon that is distinct from *Pseudoalteromonas*, with which it shares 88% sequence similarity only (Gauthier et al., 1995; Fig. 1). Intergeneric DNA-DNA similarities obtained for *Alteromonas macleodii* and species of some related genera were at the level of 5–12% (Table 2).

### Taxonomy

The genus *Alteromonas* (originally described by Baumann et al., 1972, to accommodate aerobic, Gram-negative, nonpigmented, polarly flagellated, obligately marine bacteria with DNA G+C content of 38–50 mol%) was divided into two genera: *Alteromonas* with a single species, *A. macleodii*, and *Pseudoalteromonas*, including 11 species previously belonging to *Alteromonas* (Gauthier et al., 1995). The type species is *Alteromonas macleodii*, with the type strain ATCC 27126<sup>T</sup> (= strain 107 of Baumann et al. 1972).

### Habitat

*Alteromonas macleodii* strains were isolated originally from seawater samples collected in the

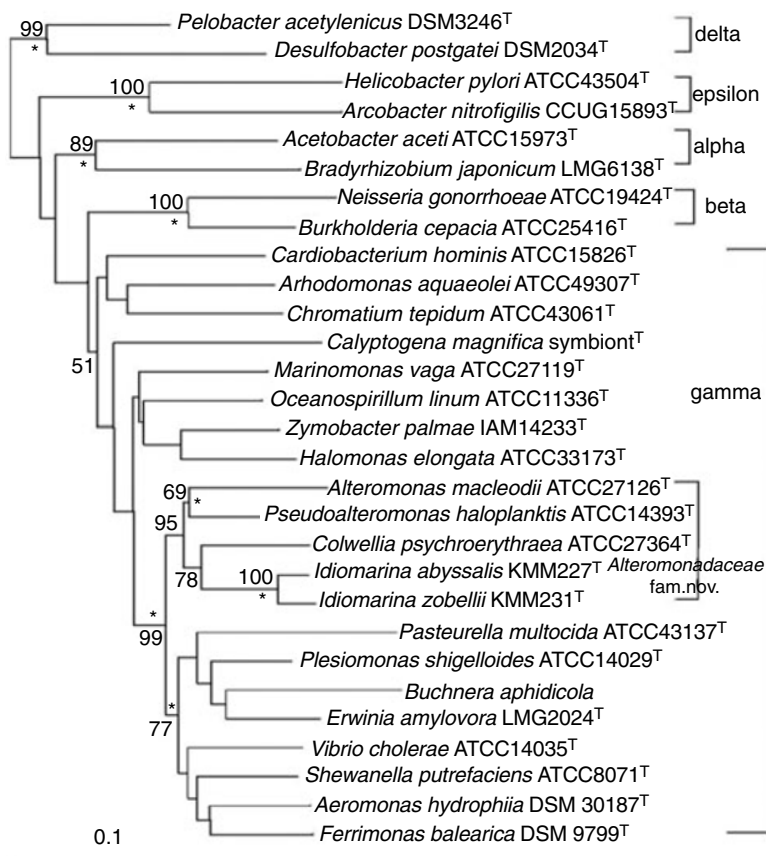


Fig. 1. Phylogenetic tree showing the position of Alteromonadaceae fam. nov. in the class Proteobacteria. Bootstrap values are given to confirm the topology of the dendrogram. Scale bar, 0.1 nucleotide substitutions per position. The accession numbers of the nucleotide sequences used for the tree construction are as follows: X70955 (*Pelobacter acetylenicus*), M26633 (*Desulfobacter postgatei*), Z25737 (*Helicobacter pylori*), L14627 (*Arcobacter nitrofigilis*), D30768 (*Acetobacter acetii*), S46916 (*Bradyrhizobium japonicum*), M86915 (*Neisseria gonorrhoeae*), L28675 (*Burkholderia cepacia*), M35014 (*Cardiobacterium hominis*), AJ000726 (*Arhodomonas aquaeolei*), M59150 (*Chromatium tepidum*), AF035730 (*Calyptogenia magnifica*), X67025 (*Marinomonas vaga*), M22365 (*Oceanospirillum linum*), D14555 (*Zymobacter palmae*), M93355 (*Halomonas elongata*), X82145 (*Alteromonas macleodii*), X67024 (*Pseudoalteromonas haloplanktis*), AF0011375 (*Colwellia psychroerythraea*), AF0552740 (*Idiomarina abyssalis*), AF052741 (*Idiomarina zobellii*), E05329 (*Pasteurella multocida*), AB025970 (*Plesiomonas shigelloides*), Z19056 (*Buchnera aphidicola*), Z96088 (*Erwinia amylovora*), X76337 (*Vibrio cholerae*), X82123 (*Shewanella putrefaciens*), X74676 (*Aeromonas hydrophila*), X93021 (*Ferrimonas balearica*).

Table 2. Levels of DNA relatedness between *Pseudoalteromonas*, *Alteromonas* and *Idiomarina* bacteria.

		DNA-DNA similarity, % (by fluorometric binding method)					
Species	Strain	G+C content, mol%	<i>P. elyakovii</i> KMM 162 <sup>T</sup>	<i>P. haloplanktis</i> IAM 12925 <sup>Ta</sup>	<i>P. carrageenovora</i> NCIMB 302 <sup>T</sup>	<i>P. espejiana</i> IAM 12640 <sup>T</sup>	<i>A. macleodii</i> IAM 12920 <sup>Ta</sup>
<i>P. haloplanktis</i>	IAM 12925 <sup>T</sup>	41.6	28.9	100	42.7	29.1	12
<i>P. nigrifaciens</i>	IAM 13010 <sup>T</sup>	40.6	ND	33	33.9	30.4	9
<i>P. elyakovii</i>	KMM 162 <sup>T</sup>	38.5	100		38.3	29.9	ND
<i>P. espejiana</i>	IAM 12640 <sup>T</sup>	41.4	29.9	21	41.2	100	12
<i>P. citrea</i>	KMM 216	43.8	49.5	35	34.2	ND	ND
<i>P. distincta</i>	KMM 638 <sup>T</sup>	43.8	52.4	30	43.6	33	10
<i>P. atlantica</i>	NCIMB 301 <sup>T</sup>	41.2	ND	32	34.7	54.5	6
<i>P. carrageenovora</i>	NCIMB 302 <sup>T</sup>	39.5	46.9	25	100	46.4	7
<i>P. antarctica</i>	CECT 4664 <sup>T</sup>	40.6–41.7	13.5	21	33 <sup>a</sup>	31 <sup>a</sup>	9
<i>P. tetraodonis</i> <sup>a</sup>	IAM 14160 <sup>T</sup>	42.1	ND	48	58	18	9
<i>P. undina</i>	IAM 12922 <sup>T</sup>	40.1	ND	54	31.6	22.4	11
<i>P. piscicida</i>	NCMB 645	43	ND	ND	13.8	10.6	5.3
<i>P. rubra</i> <sup>b</sup>	ATCC 29570 <sup>T</sup>	46–48	ND	ND	10.6	7.7	4.8
<i>A. macleodii</i> <sup>a</sup>	IAM 12920 <sup>T</sup>	46.3	ND	12	9	ND	100
<i>I. abyssalis</i> <sup>b</sup>	KMM 227 <sup>T</sup>	50.4	ND	6.0	ND	ND	7.0
<i>I. zobellii</i> <sup>b</sup>	KMM 231 <sup>T</sup>	48.0	ND	6.0	ND	ND	7.0

Symbol: <sup>T</sup>, indicates the type strain; and ND, no data available.

<sup>a</sup>Determined by initial renaturation rate method of DeLey et al. (1970).

<sup>b</sup>Data were determined by DNA-DNA dot blot hybridization method.

Data from Bozal et al. (1997), Ivanova et al. (2000b, 2001a, b) and Sawabe et al. (2000).

area of the Hawaiian archipelago (Baumann et al., 1972). *Alteromonas macleodii* subsp. *fijiensis* and *Alteromonas infernus* strains are inhabitants of deep-sea environments and have been detected in deep-seawater sample and from surface of the vestimentiferan worm *Riftia pachyptila*, which inhabits sites near hydrothermal vents (Ragueneas et al., 1996; Ragueneas et al., 1997).

## Physiology

*Alteromonas macleodii* is a Gram-negative, strictly aerobic, chemoorganotrophic, oxidase positive and catalase negative, nonsporeforming straight rod, 0.7–1 µm in diameter and 2–3 µm long, motile by single unsheathed polar flagellum, not luminescent and nonpigmented. Strains grow at 20–35°C, some strains at 37–40°C, but not at 4°C. They are able to produce extracellular amylase, gelatinase, and lipase, but not chitinase. Also, they utilize a broad range of organic compounds, including hexoses, disaccharides, sugar acids and amino acids. Strains do not denitrify and do not produce arginine dihydrolase. Poly-β-hydroxybutyrate is accumulated. Bacteria require a seawater base for growth, but not organic growth factors. The G+C content of the DNA is 44.9–46.4 mol% (Baumann et al., 1972; Baumann et al., 1984a; Baumann et al., 1984b; Gauthier et al., 1995), and that of “*Alteromonas infernus*” is 48 mol% (Ragueneas et al., 1997).

Phenotypically, *A. macleodii* strains differ from pseudoalteromonads by the ability to utilize as the sole source of carbon and energy a broad spectrum of substrates, including sucrose, cellobiose, melibiose, lactose, D-ribose, L-rhamnose, turanose, salicin, D-gluconate, DL-glycerate, L-valine and L-ornithine (Baumann et al., 1972; Baumann et al., 1984a; Gauthier et al., 1995). The main phenotypic differentiating characteristics of *Alteromonas macleodii*, *Glaciecola* and *Pseudoalteromonas* species are listed in Table 3.

## Genus *Pseudoalteromonas*

### Phylogeny

The phylogenetic structure of *Pseudoalteromonas* (Fig. 2) indicates that most of the nonpigmented species are closely related and constitute the major cluster of the genus. *Pseudoalteromonas antarctica*, *P. nigrifaciens*, *P. undina*, *P. atlantica*, *P. carrageenovora*, *P. elyakovii* and *P. distincta* are closely related to *P. haloplanktis* (99.4–99.9% sequence similarity). The pairs *P. elyakovii*–*P. distincta* and *P. haloplanktis*–*P. nigrifaciens* share almost identical 16S rDNA sequences. Also, the two pigmented species *P. citrea* and *P. aurantia* are highly related and form a separate subline, distant from *P. haloplanktis* and other nonpigmented species (97.5–97.6% sequence similarity). The recently described dark-purple pigmented species *P. ulvae* was

Table 3. Phenotypic characteristics of *Alteromonas macleodii*, *Glaciecola* and *Pseudoalteromonas* species.<sup>a</sup>

Feature	<i>Alteromonas macleodii</i> <sup>b</sup>	<i>Glaciecola punicea</i> <sup>c</sup>	<i>Glaciecola pallidula</i> <sup>c</sup>	<i>Pseudoalteromonas</i>
Flagella arrangement	Polar, 1	ND	ND	Polar, 1; or tuft of 2–4; lateral
Filaments presence	–	+	+	V
Pigmentation	None	Pink-red	Pale pink	V–
Growth at				
0°C	–	+	+	–
4°C	–	+	+	V+
25°C	+	+	–	+
35°C	+	–	–	V
37°C	+	–	–	–
40°C	V	–	–	–
Hydrolysis of				
Urea	ND	–	–	V
Gelatin	+	–	–	+
Esculin	ND	V+	–	V
Starch	+	–	V+	V
Agar	–	–	–	V
Alginate	V	–	–	V
Chitin	–	–	–	V
Tween-80	+	V–	+	+
DNA	+	–	–	+
Acids production from				
D-Glucose	+	W	W	V
D-Galactose	+	W	W	V
D-Melibiose	V	W	W	V
Glycerol	+	W	W	V
Maltose	+	–	W	V
Utilization of				
D-Glucose	+	–	–	V
D-Galactose	+	–	–	V
D-Fructose	+	–	–	V
D-Mannose	–	–	–	V
D-Xylose	V	–	–	V
Sucrose	+	–	–	V
Glycerol	+	–	+	V
Citrate	–	–	–	V
Acetate	+	–	+	V
DL-Lactate	V	–	+	V
L-Tyrosine	+	V+	–	V
G+C content, mol%	44–47	44–46	40	37–50

Symbols: +, positive test reaction; –, negative test reaction; V, variable results between species; V+, variable results between strains, type strain is positive; V–, variable results between strains, type strain is negative; W, weak or delayed reaction; and ND, not determined.

<sup>a</sup>All strains were positive for the following tests: motility, sodium ions requirement for growth, oxidase, growth in 1–3% NaCl, growth at 7–20°C; all strains were negative for arginine dihydrolase production.

<sup>b</sup>Data from Baumann et al. (1984a, b).

<sup>c</sup>Data from Bowman et al. (1998c).

found to show 97% similarity to other *Pseudoalteromonas* species (Egan et al., 2001). *Pseudoalteromonas prydzensis* forms a subcluster distinct from both the nonpigmented and pigmented species (97.3–97.7% and 95.0–95.8% sequence similarity, respectively). *Pseudoalteromonas peptidolytica* is most similar to *P. piscicida* (99.1% sequence similarity), which, together with *P. luteoviolacea* and *P. rubra*, constitute a separate subcluster among the pigmented *Pseudoalteromonas* species. *Pseudoalteromonas*

*denitrificans* and *P. tunicata* constitute individual deep clades within the genus *Pseudoalteromonas*. With 90.6–91.0% sequence similarities with other *Pseudoalteromonas* species, *P. bacteriolytica* constitutes the deepest branch within the genus.

Also included in Fig. 2 is the phylogenetic position of novel *Pseudoalteromonas* species, including *P. ruthenica*, *P. flavipulchra*, *P. maricaloris*, *P. issachenkonii* (Ivanova et al., 2002a; Ivanova et al., 2002b; Ivanova et al., 2002c), *P.*

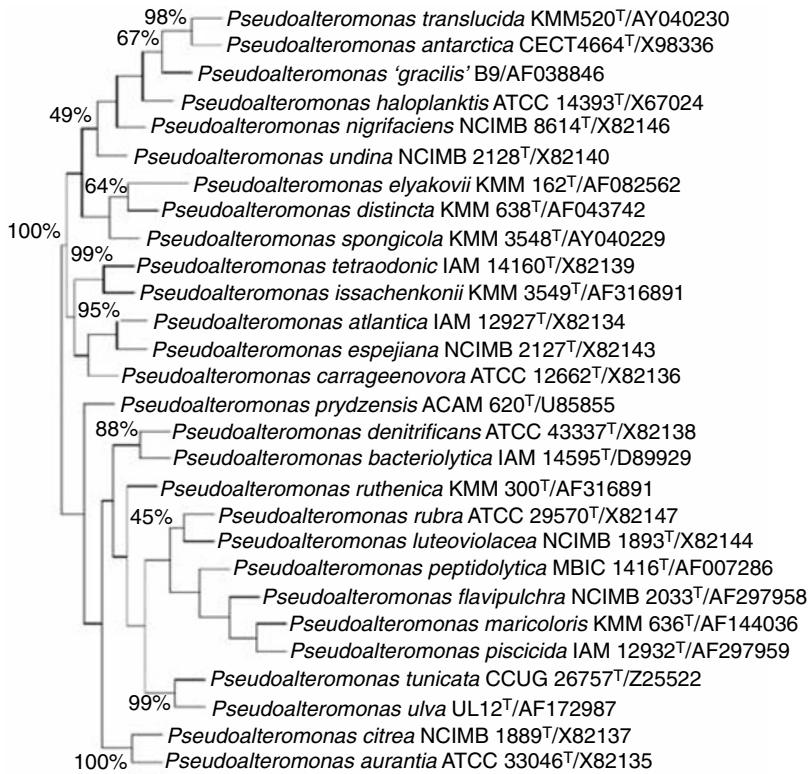


Fig. 2. Phylogenetic structure of the genus *Pseudoalteromonas*. Bootstrap values are given to confirm the topology of unrooted dendrogram.

*paragorgicola* and *P. translucida* which will be published in the near future.

### Taxonomy

At the present time, *Pseudoalteromonas* genus contains 27 validly described species, including reclassified former *Alteromonas* species, listed in Table 1, and recently described *Pseudoalteromonas* species (Bozal et al., 1997; Bowman, 1998a; Holmstrom et al., 1998; Sawabe et al., 1998a; Venkateswaran and Dohmoto, 2000; Egan et al., 2001; Ivanova et al., 2002a; Ivanova et al., 2002b; Ivanova et al., 2002c).

### Habitat

Bacteria of the genus *Pseudoalteromonas* are obligate and widespread inhabitants of marine environments and have been isolated from diverse marine sources from various geographical regions of the world's oceans (Table 4).

### Physiology

*Pseudoalteromonas* strains are aerobic, Gram-negative, nonsporeforming, straight or slightly curved, rod-shaped bacteria 0.2–1.5  $\mu\text{m} \times$  1.8–4.0  $\mu\text{m}$ . Strains of most species are motile by means of a single unsheathed polar flagellum.

Sheathed flagella have been reported for *P. luteoviolacea*, *P. denitrificans* (Novic and Tyler, 1985; Enger et al., 1987) and *P. tunicata* (Holmstrom et al., 1998). *Pseudoalteromonas denitrificans* may produce a tuft of 2–3 flagella at one pole. *Pseudoalteromonas distincta* is characterized by both a single polar and 3–7 lateral flagella (Fig. 3a). Cells of *P. antarctica* have been observed to form filaments up to 10  $\mu\text{m}$  long (Bozal et al., 1997). Peritrichous flagellation had been originally noted for *P. piscicida* (Bein, 1954). The bacteria of *P. nigrifaciens* (Fig. 3b, c) and *P. elyakovii* (Fig. 3d) are able to form capsules (Ivanova et al., 1996a; Ivanova et al., 1996b; Sawabe et al., 2000).

The bacteria are oxidase positive, and catalase positive or weakly positive. They do not accumulate poly- $\beta$ -hydroxybutyrate and show no arginine dihydrolase activity. *Pseudoalteromonas* are chemoorganotrophs with a respiratory type of metabolism. *Pseudoalteromonas tunicata* and *P. ulvae* are described as a facultative anaerobic bacteria (Holmstrom et al., 1998; Egan et al., 2001). *Pseudoalteromonas denitrificans* is able to denitrify (Enger et al., 1987). *Pseudoalteromonas denitrificans* and only some *P. haloplanktis* strains form nitrite from nitrates. Some pigmented species need additional organic factors for growth. All species grow at 20–25°C. Most species are able to grow at 4°C. Growth from 0

Table 4. Marine bacteria of the *Alteromonas* and related genera.

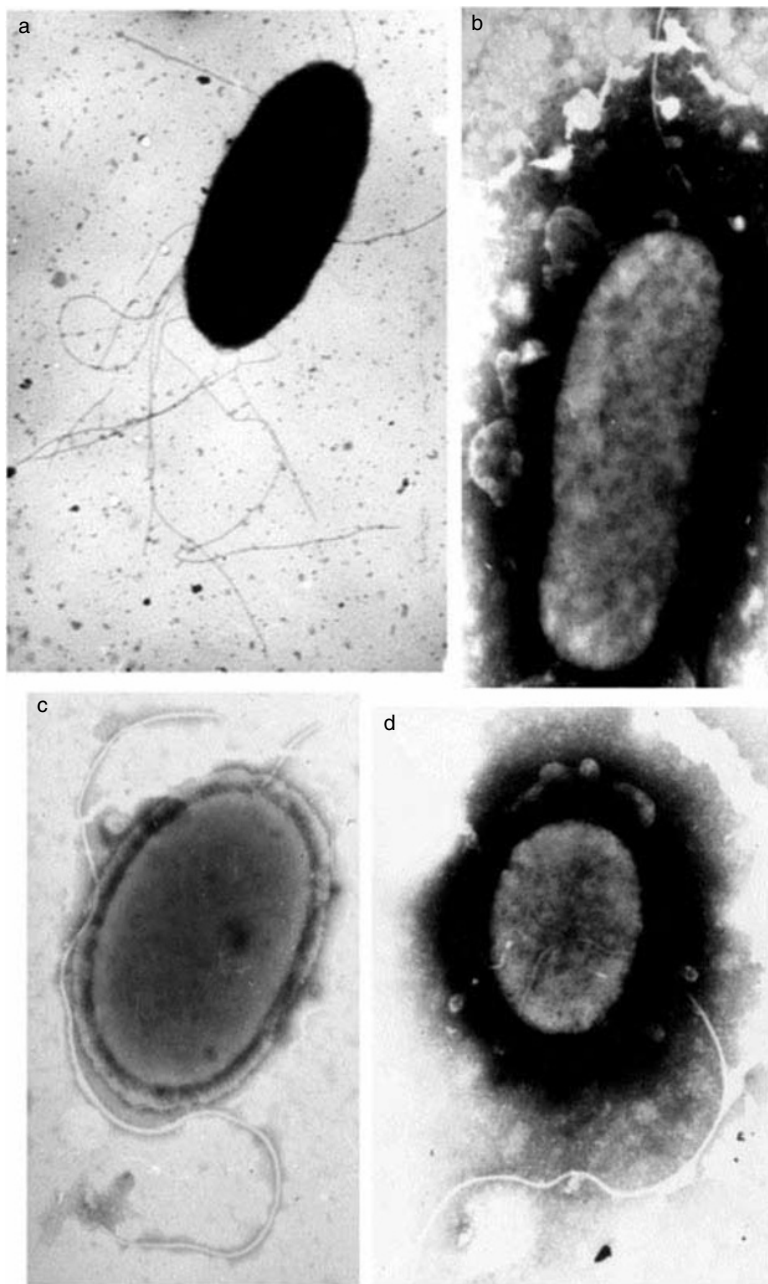
Species (type strain)	Production of biologically active substances	Isolation source	Isolation place	References
<i>Alteromonas macleodii</i> (ATCC 27126 <sup>1</sup> )		Seawater	Hawaiian archipelago	Baumann et al., 1972
" <i>Alteromonas macleodii</i> subsp. <i>fijiensis</i> " (CNCM I-1627 <sup>1</sup> )	High-molecular polysaccharide	Hydrothermal vents, 2000m	Fiji Islands	Raguenes et al., 1996
" <i>Alteromonas infernus</i> " (CNCM I-1628 <sup>1</sup> )	High-molecular polysaccharide	Hydrothermal vents, 2000m	Guayama reefs	Raguenes et al., 1997
<i>Glaciecola pallidula</i> (ACAM 615 <sup>1</sup> )		Sea ice diatom assemblages	Antarctica	Bowman et al., 1998c
<i>Glaciecola punicea</i> (ACAM 611 <sup>1</sup> )		Sea ice diatom assemblages	Antarctica	Bowman et al., 1998c
<i>Pseudoalteromonas antarctica</i> (CECT 4664 <sup>1</sup> )	Polysaccharide	Muddy soils and sediments	Antarctic coastal areas	Bozal et al., 1997
<i>Pseudoalteromonas atlantica</i> (ATCC 19262 <sup>1</sup> )	Agarases	Seaweeds	Atlantic and Pacific coastal areas	Akagawa-Matsushita et al., 1992b
<i>Pseudoalteromonas aurantia</i> (ATCC 33046 <sup>1</sup> )	Antibiotics, autotoxins	Surface of stones, algae, and fish; sediments; and coastal seawater	French coast of Mediterranean Sea	Gauthier and Breittmayer, 1979
Species (type strain)	Production of biologically active substances	Isolation source	Isolation place	References
<i>Pseudoalteromonas bacteriolytica</i> (IAM 14595 <sup>1</sup> )	Bacteriolytic activity; prodigiosin-like pigment	Red-spotted beds of <i>Laminaria japonica</i>	The Sea of Japan	Sawabe et al., 1998a
<i>Pseudoalteromonas carrageenovora</i> (ATCC 43555 <sup>1</sup> )	Carrageenases	Seaweeds	Pacific coastal waters	Akagawa-Matsushita et al., 1992b
<i>Pseudoalteromonas citrea</i> (ATCC 29719 <sup>1</sup> )	Antibiotics, autotoxins	Surface of stones, algae, and fish; sediments; and coastal seawater	French coast of Mediterranean Sea; and Russian coast of the Sea of Japan	Gauthier, 1977
<i>Pseudoalteromonas denitrificans</i> (ATCC 43337 <sup>1</sup> )	Prodigiosin production; antibiotic activity	Seawater, 90–100m	West coast of Norway (fjords)	Ivanova et al., 1998b
<i>Pseudoalteromonas distincta</i> (KMM 638 <sup>1</sup> )	Melanin-like pigment	Sea sponge	Pacific Ocean, Komandorskie Islands	Enger et al., 1987
<i>Pseudoalteromonas elyakovii</i> (KMM 162 <sup>1</sup> )	$\beta$ -1,3-gluconase, alkaline phosphatase; alginate lyase and alginate-degrading enzymes	Mussel <i>Crenomytilus grayanus</i> , spot-wounded fronds of <i>Laminaria japonica</i>	The Sea of Japan	Ivanova et al., 2000a
<i>Pseudoalteromonas espejiana</i> (ATCC 29659 <sup>1</sup> )	Nuclease	Seawater	Eastern coast of North America, and Chile	Ivanova et al., 1996b Sawabe et al., 2000 Chan et al., 1978
Species (type strain)	Production of biologically active substances	Isolation source	Isolation place	References
<i>Pseudoalteromonas haloplanktis</i> (ATCC 14393 <sup>1</sup> )	$\beta$ -galactosidase	Coastal and open ocean seawater; damaged tissues of fishes and oysters; and deep-sea mud	East and west coasts of North America, Indian Ocean, Hawaiian archipelago, and the Sea of Japan	Baumann et al., 1972 Colwell and Sparks, 1967 Kameyama et al., 1987 Reichelt and Baumann, 1973 ZoBell and Upham, 1944

Table 4. Continued

Species (type strain)	Production of biologically active substances	Isolation source	Isolation place	References
<i>Pseudoalteromonas luteoviolacea</i> (ATCC 33492 <sup>1</sup> )	Violacein production; antibiotic and autotoxic activity	Surface of stones, algae, and animals; marine sediment and coastal waters	French coast of Mediterranean Sea; and the Sea of Japan	Gauthier, 1976b, 1982 Kakimoto et al., 1980
<i>Pseudoalteromonas nigrifaciens</i> (ATCC 19375 <sup>1</sup> )	Unusual acidic polysaccharides, and melanin production	Sea water; marine animals; the type strain was isolated from spoiled salt butter	Russian coast of the Sea of Japan	White, 1940
<i>Pseudoalteromonas peptidolytica</i> (MBICC F1250A1 <sup>1</sup> )	Proteases degrading marine mussel thread	Surface seawater	The Sea of Japan	Ivanova et al., 1996a Venkateswaram and Dohmoto, 2000
<i>Pseudoalteromonas piscicida</i> (ATCC 15057 <sup>1</sup> )	Antibiotics, and toxins	Zones of "red tide" and surface of wounded fishes	Southwestern coast of Florida	Bein, 1954
<i>Pseudoalteromonas prydzensis</i> (ACAM 620 <sup>1</sup> )		Sea ice	Prydz Bay, Antarctica	Bowman, 1998a
Production of biologically active substances				
Species (type strain)	Production of biologically active substances	Isolation source	Isolation place	References
<i>Pseudoalteromonas rubra</i> (ATCC 29570 <sup>1</sup> )	Prodigiosin production; antibiotics, and autotoxins	Surface of stones, algae, and animals; marine sediment and coastal waters	French coast of Mediterranean Sea	Gauthier, 1976a
<i>Pseudoalteromonas tetraodonis</i> (IAM 14160 <sup>1</sup> )	Tetrodotoxin	Skin slime of the fish <i>Fugu poecilonotus</i>	Sea of Japan	Simidu et al., 1990 Akagawa-Matsushita et al., 1993 Ivanova et al., 2001c
<i>Pseudoalteromonas tunicata</i> (CCUG 26757 <sup>1</sup> )	Antifouling agents production	Tunicate <i>Ciona intestinalis</i>	Western Sweden coast	Holmstrom et al., 1998
<i>Pseudoalteromonas ubvae</i> (UNSW 095600 <sup>1</sup> )	Antifouling agents production	Surface of marine alga <i>Ulva lactuca</i>	East coast of Australia	Egan et al., 2001
<i>Pseudoalteromonas undina</i> (ATCC 29660 <sup>1</sup> )		Seawater	Eastern coast of North America	Chan et al., 1978
<i>Idiomarina abyssalis</i> (KMM 227 <sup>1</sup> )		Deep-sea water, 4000 m	Pacific Ocean	Ivanova et al., 2000b
<i>Idiomarina zobelii</i> (KMM 231 <sup>1</sup> )		Deep-sea water, 5000 m	Pacific Ocean	Ivanova et al., 2000b
<i>Cobwellia demingiae</i> (ACAM 459 <sup>1</sup> )		Sea ice	Antarctica	Bowman et al., 1998b
<i>Cobwellia hadaliensis</i> BNL 1 <sup>T</sup>		Deep-sea water, 7410 m	Puerto Rico Trench	Deming et al., 1988
Production of biologically active substances				
Species (type strain)	Production of biologically active substances	Isolation source	Isolation place	References
<i>Cobwellia hornerae</i> (ACAM 607 <sup>1</sup> )		Sea ice	Antarctica	Bowman et al., 1998b
<i>Cobwellia maris</i> (JCM 10085 <sup>1</sup> )		Sea water	The coast of the Sea of Japan, Hokkaido	Yumoto et al., 1998
<i>Cobwellia psychrethraea</i> (ATCC 27364 <sup>1</sup> )		Deep-sea water, 6000 m; sea ice	Pacific Ocean, Antarctica	Deming et al., 1988 Bowman et al., 1998b
<i>Cobwellia psychrotropica</i> (ACAM 179 <sup>1</sup> )		Deep-sea water and sea ice	Antarctica	Bowman et al., 1998b
<i>Cobwellia rossensis</i> (ACAM 608 <sup>1</sup> )		Sea ice	Antarctica	Bowman et al., 1998b
<i>Thalassomonas viridans</i> (CECT 5083 <sup>1</sup> )		Oysters	Mediterranean coast, Spain	Macian et al., 2001



Fig. 3. Transmission electronic micrographs of *Pseudoalteromonas distincta* KMM 638<sup>T</sup> (a), *Pseudoalteromonas nigrifaciens* KMM 158 (b), KMM 156 (c), and *Pseudoalteromonas elyakovii* KMM 162<sup>T</sup> (d). (a) Cell with polar and lateral flagella,  $\times 20,000$ ; (b, c, and d) Cells with single polar flagellum and capsules, b, d,  $\times 30,000$ , and c,  $\times 40,000$ .



to 30°C was noted for *P. prydzensis* (Bowman, 1998a). *Pseudoalteromonas aurantia*, *P. undina*, *P. prydzensis*, *P. denitrificans*, *P. antarctica*, *P. tunicata*, *P. ulvae*, some strains of *P. nigrifaciens* and of *P. espejiana* do not grow at 35°C. Several species such as *P. piscicida*, *P. elyakovii* (some strains only) and *P. peptidolytica* grow at 37°C. Except for *P. peptidolytica* (Venkateswaran and Dohmoto, 2000), none of the species grows at 40°C.

Several studies have shown that sodium ions are essential for alteromonad-like bacteria

(MacLeod and Hori, 1960; MacLeod, 1968). Considering the marine origin and sodium ion requirement for growth, *Pseudoalteromonas* strains could be regarded as obligately marine microorganisms. All species need seawater or sodium ions for growth and the most of them grow in 3–6% NaCl. For *P. ulvae*, 1–2% NaCl is required for growth (Egan et al., 2001). Several species are able to grow in 10% NaCl. *Pseudoalteromonas antarctica* and *P. prydzensis* tolerate up to 12.5 and 15% NaCl, respectively. The genus consists mainly of species isolated

from marine environments, and they have not been detected from terrestrial and some other sources with the exception of the *P. nigrifaciens* type strain, isolated from salted butter (White, 1940).

Pigmented species are able to produce intracellular insoluble noncarotenoid pigments and melanin-like pigments (Table 4) and synthesize high molecular weight substances with autotoxic and antibiotic effects against Gram-positive and Gram-negative bacteria. The antibiotic substance from cells of *P. rubra* has been shown to be a complex membrane-bound glycoprotein (Ballester et al., 1977). *Pseudoalteromonas citrea* produces two polyanionic antibiotic compounds (Gauthier, 1977). Two brominated antibacterial products and polyanionic polysaccharides inhibiting the growth of Gram-positive, and to lesser extent, Gram-negative bacteria, as well as the protozoan *Paramecium caudatum*, were isolated from *P. luteoviolacea* (Gauthier and Flatau, 1976c; Kamei et al., 1986).

Pseudoalteromonads utilize a variety of organic compounds as sole sources of carbon and energy, including carbohydrates, monocarboxylic acids and amino acids, and it should be noted that utilization of carbon sources varies between species and strains within some species (Tables 5 and 6). The majority of *Pseudoalteromonas* species is characterized by high hydrolytic activities and produces gelatinase, lipase, caseinase, lecithinase and DNAses; they utilize D-glucose as a sole carbon source, but are negative for utilization of D-ribose, L-rhamnose, turanose, salicin, glucuronate, DL-glycerate, erythritol, sorbitol, *meso*-inositol, adonitol, L-valine, L-ornithine and *m*-hydroxybenzoate. *Pseudoalteromonas distincta* does not utilize D-glucose and other carbohydrates. Some species display amylase, alginase and chitinase activities. *Pseudoalteromonas atlantica* and *P. carrageenovora* (Akagawa-Matsushita et al., 1992b) and some strains of *P. citrea* (Ivanova et al., 1998b) are able to degrade agar and/or carrageenan. Most members of *Pseudoalteromonas* are resistant to the O/129 vibriostatic agent (2,4-diamino-6,7-diisopropyl pteridine sulfate or phosphate; (Gauthier and Breittmayer, 1992) and also to penicillin, polymyxin, lincomycin and oleandomycin (Ivanova et al., 2001b). Penicillin-sensitive *P. antarctica* strains were noted by Bozal et al. (1997); *P. tunicata* and *P. ulvae* are susceptible to O/129 (Holmstrom et al., 1998; Egan et al., 2001).

The phenotypic features of the new *Pseudoalteromonas* species, *P. ruthenica*, *P. flavipulchra*, *P. maricaloris*, *P. paragorgicola*, *P. translucida* (Ivanova et al., 2002a, b, c) and *P. issachenkonii* and *P. translucida* which will be published in the near future, are presented in Table 6.

## Genomic Relatedness

The G+C contents in the DNA of *Pseudoalteromonas* bacteria range from 37 mol% (*P. denitrificans*) to 44–46 mol% (*P. bacteriolytica*) and 46–48 mol% (*P. rubra* and *P. ruthenica*; Tables 5 and 6). DNA-DNA hybridization studies revealed interspecies relatedness between most of the *Pseudoalteromonas* species at level of 20–40% (Table 2). Though some species revealed higher DNA-DNA reassociation, namely, *P. haloplanktis* and *P. undina* (54%), *P. espejiana* and *P. atlantica* (54%), *P. tetraodonis* and *P. carrageenovora* (58%), *P. distincta* and *P. elyakovii* (61%). *Pseudoalteromonas prydzensis* had low DNA-DNA reassociation levels (10–28%) with the other nonpigmented pseudoalteromonads (Bowman, 1998a). Supporting low 16S rDNA similarities, *P. bacteriolytica* and *P. peptidolytica* were most distantly related (2–6% DNA-DNA relatedness as determined by using a fluorometric direct binding method) to the pigmented *Pseudoalteromonas* species (Sawabe et al., 1998a; Venkateswaran and Dohmoto, 2000).

## Genus *Glaciecola*

### Phylogeny

The genus *Glaciecola* forms a separate 16S rDNA lineage within the  $\gamma$ -subclass of Proteobacteria, and is distantly related to *Alteromonas macleodii* (89.7–92.1% sequence similarity; Bowman et al., 1998c).

### Taxonomy

The genus *Glaciecola* was proposed by Bowman et al. (1998c) for two groups of isolates originating from sea-ice diatom assemblages collected from coastal area of eastern Antarctica. The genus is defined as rod-shaped or slightly curved, curved or spiral cells which are Gram negative, oxidase- and catalase positive, motile, nonsporulating, strictly aerobic, chemoheterotrophic, psychrophilic and slightly halophilic, and require seawater for growth. Major fatty acids are hexadecaenoic acid (16:1 $\omega$ 7c), hexadecaenoic acid (16:0) and octadecaenoic acid (18:1 $\omega$ 7c). The DNA G+C content is 40–46 mol% (determined by the thermal denaturation method). The type species is *Glaciecola punicea* ACAM 611<sup>T</sup>.

The *Glaciecola* genus comprises two species, the type species *Glaciecola punicea*, type strain ACAM 611<sup>T</sup>, and *Glaciecola pallidula*, type strain ACAM 615<sup>T</sup>, which differ from each other in phenotypic features (Table 7), DNA-DNA hybridization, and phylogenetic data. The fatty acid profiles of *Glaciecola* strains are very similar

Table 5. Phenotypic properties of *Pseudoalteromonas* species.<sup>a,b</sup>

Property	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
DNA G+C content, mol%	38-43	44-46	38.9-44.7	36.5-37.1	40-43	37.9	43-46	46-48	42-43	41-42	40.6-41.7	39.5	43.8	38-39	43-44	41.8-44.4	39-41.7	38-39	41.5	43-44	ND	
Flagellation																						
Polar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lateral												+	+									
Pigmentation																						
Melanin-like													+									
Other	+	+	+	+	+	+	+	+	+								+					+
Growth at																						
4°C	+	-	V	+	V	-	-	-	+	+	-	-	+	V	-	-	V	+	+	V	+	-
-37°C	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Growth in NaCl																						
1%	-	+	+	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
3-6%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
8%	-	-	+	-	-	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	-
10%	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	+	+	+	+	-
12%	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	+	+	+	+	-
Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
15%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of																						
Amylase	+	+	+	+	+	+	-	+	ND	-	+	-	-	+	+	V	+	+	-	V	ND	
Caseinase	ND	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	ND	
Alginate	-	-	V	+	ND	+	ND	ND	ND	ND	+	+	-	+	+	-	+	-	ND	-	ND	
Agarase	-	-	V	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	
Chitinase	-	-	-	+	-	-	-	-	ND	-	-	-	-	-	-	V	-	+	-	+	ND	
Utilization of																						
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
L-Arabinose	-	+	-	-	-	ND	-	-	-	-	-	-	-	ND	-	V	V	+	-	-	-	-
D-Mannose	+	+	+	ND	-	-	+	+	+	V	+	-	-	+	V	+	+	+	-	-	-	+

Table 5. Continued

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
D-Galactose	-	+	-	-	-	-	ND	-	-	+	+	+	-	+	+	V	+	+	+	-	ND
D-Fructose	+	+	+	-	-	-	-	-	-	-	+	+	-	+	V	V	+	-	-	-	-
Maltose	V	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+
Sucrose	-	+	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	-
Melibiose	-	-	-	ND	-	ND	-	-	-	-	+	+	-	+	+	-	+	-	-	-	-
Lactose	-	-	+	-	-	-	-	-	-	+	+	+	-	+	+	-	+	-	-	-	-
D-Gluconate	-	-	+	-	-	ND	ND	-	ND	V	-	-	ND	-	-	-	+	+	-	-	ND
Glycerol	-	-	-	-	-	-	-	-	-	ND	+	+	-	-	-	-	V	+	-	-	-
Succinate	-	+	+	-	-	+	ND	-	ND	+	+	+	ND	+	-	+	V	+	ND	+	ND
D-Mannitol	-	V	-	-	-	-	-	-	-	+	+	+	-	+	+	V	V	+	-	-	ND
Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Citrate	-	-	-	V	-	-	+	-	-	+	-	+	+	V	+	+	-	+	+	-	+
N-Acetylglucosamine	+	-	ND	-	+	+	ND	-	ND	V	-	-	-	-	-	+	ND	+	ND	+	ND
Fumarate	-	+	-	-	-	+	ND	-	-	+	+	+	-	+	+	+	+	+	ND	+	ND
Xylose	-	-	-	ND	-	ND	-	-	-	ND	-	-	-	+	-	-	+	-	-	-	-
Trehalose	+	+	+	ND	+	ND	ND	+	+	ND	+	-	-	-	+	V	+	+	-	+	-
Acetate	-	+	+	+	-	-	ND	-	ND	-	+	+	ND	+	+	+	+	+	+	+	ND
Pyruvate	-	+	+	+	V	ND	ND	-	ND	+	+	+	ND	+	+	+	+	+	+	-	ND
L-Tyrosine	+	V	+	+	V	ND	ND	ND	ND	ND	-	-	+	-	+	+	+	V	+	+	ND
L-Arginine	+	ND	-	+	V	ND	ND	-	ND	ND	-	-	+	ND	V	V	+	ND	-	V	ND

Symbols: +, positive; -, negative; V, variable reaction between strains; and ND, no data available.

<sup>a</sup>Species: 1, *P. aurantia*; 2, *P. bacteriolytica*; 3, *P. citrea*; 4, *P. denitrificans*; 5, *P. luteoviolacea*; 6, *P. peptidolytica*; 7, *P. piscicida*; 8, *P. rubra*; 9, *P. tuniaca*; 10, *P. antarctica*; 11, *P. atlantica*; 12, *P. carraegenovora*; 13, *P. distincta*; 14, *P. elyakovii*; 15, *P. espejiana*; 16, *P. haloplanktis*; 17, *P. nigrifaciens*; 18, *P. prydzensis*; 19, *P. tetraodonis*; 20, *P. undina*; and 21, *P. ulvae*.

<sup>b</sup>All strains require sodium ions for growth, positive for oxidase, catalase, and gelatin hydrolysis.

Based on species description data were obtained from: Bein (1954), Gauthier (1976a, b), Gauthier and Breittmayer (1979), Baumann et al. (1984b), Enger et al. (1987), Akagawa-Matshusita et al. (1992b), Bozal et al. (1997), Bowman (1998a), Holmstrom et al. (1998), Ivanova et al. (1996a, b, 2000a, 2001c), Sawabe et al. (1998a, 2000), Venkateswaran and Dohmoto (2000), and Egan et al. (2001).

Table 6. Phenotypic features of the new *Pseudoalteromonas* species.<sup>a,b</sup>

Characteristic	<i>P. paragorgicola</i> KMM 3548 <sup>T</sup>	<i>P. flavipulchra</i> NCIMB 2033 <sup>T</sup>	<i>P. maricaloris</i> KMM 636 <sup>T</sup>	<i>P. ruthenica</i> KMM 300 <sup>T</sup>	<i>P. translucida</i> KMM 520 <sup>T</sup>	<i>P. issachenkonii</i> KMM 3549 <sup>T</sup>
G+C mol%	41.1	41.7	39.1	48.4	46.3	43
Flagellation						
Polar	+	+	+	+	+	+
Bipolar						
Pigmentation						
Melanin-like					–	–
Other	+	+	+	+		
Growth at						
4°C	+	–	–	–	+	+
37°C	–	+	+	+	–	+
Growth in NaCl						
1%	+	+	+	+	+	+
8%	–	+	+	+	+	+
10%	–	+	+	–	–	+
12%	–	+	–	–	–	+
15%	–	–	–	–	–	+
Production of						
Amylase	+	+	+	+	+	–
Caseinase	+	+	+	+	+	+
Alginase	ND	ND	+	+	+	+
Agarase	–	–	–	–	–	–
Chitinase	–	+	–	–	–	+
DNase	+	+	+	+	+	+
Utilization of						
D-Glucose	ND	+	+	+	–	+
D-Arabinose	+	–	+	–	+	ND
D-Mannose	ND	+	+	–	ND	–
D-Galactose	+	–	+	–	+	+
D-Fructose	ND	+	+	–	ND	+
Maltose	+	+	+	–	–	+
Sucrose	ND	+	+	–	ND	+
Melibiose	–	–	+	–	–	+
Lactose	–	–	+	–	–	+
D-Gluconate	ND	–	–	–	ND	–
Glycerol	ND	–	+	–	ND	–
Succinate	ND	+	–	–	ND	+
D-Mannitol	+	–	+	–	+	+
Sorbitol	–	–	+	–	ND	–
Citrate	–	+	+	–	–	+
<i>N</i> -Acetylglucosamine	ND	–	–	–	ND	–
Fumarate	ND	+	–	–	ND	+
Xylose	ND	–	–	–	–	–
Trehalose	ND	–	+	–	ND	–
Acetate	ND	–	–	–	ND	+
Pyruvate	ND	+	–	–	ND	+
L-Tyrosine	–	+	–	–	ND	ND
L-Arginine	–	–	+	–	–	ND
Susceptibility to						
Ampicillin (10µg)	+	–	–	–	–	+
Kanamycin (30µg)	–	+	V	V	–	–
Carbenicillin (100 ED)	+	+	ND	+	–	+
Lincomycin (15µg)	–	–	–	–	–	–
Oleandomycin (15µg)	–	–	+	–	–	+
Polymyxin (300 ED)	+	+	+	+	+	+
Streptomycin (10µg)	+	V	+	–	+	+
Tetracycline (30µg)	–	–	–	+	–	+

Symbols: +, positive; –, negative; V, strain variation; and ND, no data available.

<sup>a</sup>*P. flavipulchra*, *P. maricaloris*, *P. ruthenica*, and *P. issachenkonii* (Ivanova et al., 2002a, b, c); data for *P. translucida* and *P. paragorgicola* from (Ivanova et al., in press).

<sup>b</sup>All strains studied require sodium ions for growth, positive for oxidase and catalase, for hydrolysis of gelatin, Tween 80, growth at 3–6% NaCl, susceptible to gentamicin, and resistance to benzylpenicillin.

Table 7. Phenotypic characteristics differentiating *G. punicea* and *G. pallidula*.<sup>a</sup>

Characteristic	<i>G. punicea</i> (n <sup>b</sup> = 15)	<i>G. pallidula</i> (n = 3)
Pigmentation	Pink-red	Pale pink
Yeast extract requirement	V+	–
β-Galactosidase-6-phosphatase	+	–
Hydrolysis of		
Urate	V–	–
Esculin	V+	–
Starch	–	V+
Tween 80	V–	+
Acid from maltose	–	(+)
Utilization of		
α-Glycerophosphate	V+	+
Succinate, L-malate, fumarate, and L-proline	+	–
Glycerol, glycogen, acetate, pyruvate, DL-lactate, and L-glutamate	–	+
L-Tyrosine	V+	–
Butyrate	–	V–
G+C content (mol%) <sup>c</sup>	44–46	40

Symbols: +, test positive for all strains; V+, variable results between strains, type strain positive; V–, variable results between strains, type strain negative; –, all strains negative for test; and (+), weak and delayed reaction.

<sup>a</sup>All strains were positive for the following tests: growth at 0–10°C; growth on marine 2216 agar, R2A seawater agar and ZoBell's agar; production of catalase, cytochrome-*c* oxidase; α-galactosidase and β-galactosidase; acid production (weak and/or delayed) from D-glucose, D-galactose, D-melibiose and glycerol; and utilization of oxaloacetate as a sole carbon and energy source. All strains were negative for the following tests: growth at 25°C and higher; growth with 3× and 4× strength seawater, tolerance to 5% ox bile salts; nitrate as a nitrogen source; nitrate reduction, denitrification and nitrogen fixation; hydrolysis of urea, xanthine, egg yolk, casein, gelatin, chitin, dextran and DNA; production of L-phenylalanine deaminase, L-tryptophan deaminase, arginine dihydrolase, glutamate decarboxylase, lysine decarboxylase, ornithine decarboxylase, α-glucosidase, β-glucosidase, α-fucosidase, α-arabinosidase, β-glucuronidase, β-N-acetylglucosaminidase; indole from L-tryptophan; hydrogen sulfide from thiosulfate; acid production from L-arabinose, D-mannose, D-fructose, L-rhamnose, D-xylose, D-mannitol, N-acetylglucosamine, sucrose, lactose, cellobiose, trehalose, D-raffinose, dextran, adonitol, L-arabitol, D-sorbitol and *m*-inositol; utilization of N-acetylglucosamine, L-arabinose, cellobiose, D-xylose, L-rhamnose, D-fructose, D-galactose, D-glucose, D-mannose, lactose, maltose, D-melibiose, D-raffinose, sucrose, trehalose, starch, chitin, adonitol, L-arabitol, *i*-erythritol, *m*-inositol, D-mannitol, D-sorbitol, D-gluconate, D-glucuronate, saccharate, propionate, isobutyrate, valerate, isovalerate, caproate, heptanoate, nonanoate, malonate, adipate, glutarate, pimelate, azelate, *trans*-aconitate, citrate, 3-hydroxybutyrate, 2-oxoglutarate, L-alanine, L-asparagine, L-aspartate, L-histidine, L-leucine, L-phenylalanine, hydroxy-L-proline, L-ornithine, L-serine, L-threonine, putrescine, γ-aminobutyrate and urate.

<sup>b</sup>Numbers of strains studied.

<sup>c</sup>Measured by T<sub>m</sub>.

Data from Bowman et al. (1998c).

to the whole-cell fatty acid profile of *Alteromonas macleodii*, but substantially more enriched in monounsaturated fatty acids (Bowman et al., 1998c; Table 8).

## Habitat

Antarctic sea-ice diatom assemblages are the only known habitat (Bowman et al., 1998c).

## Physiology

*Glaciecola* strains are psychrophilic, able to grow at temperature from –2 to 20°C. Most *G. punicea* strains grow slowly at 25°C but not at higher temperatures. *Alteromonas* species, the closest relatives, have a mesophilic growth temperature range, are more halotolerant, and versatile in

respect to carbon source utilization and extracellular enzyme production (Table 3). *Glaciecola* strains are distinguished from the other marine chemoheterotrophs belonging to the γ-Proteobacteria including *Colwellia*, *Pseudoalteromonas*, *Alteromonas* and *Shewanella* by phenotypic features (Table 9).

## Genomic Relatedness

The DNA G+C contents of *G. punicea* and *G. pallidula* strains are 44–46 mol% and 40 mol%, respectively. *Glaciecola punicea* strains exhibit high DNA-DNA reassociation levels of 82–95% among each other, and low DNA reassociation with strains of *G. pallidula* (10–19%). DNA-DNA hybridization levels between *A. macleodii*, *G. punicea* and *G. pallidula* strains were 13 and 10%, respectively (Bowman et al., 1998c).

Table 8. Fatty acid compositiona of *Glaciecola* species and *Alteromonas macleodii*.

Fatty acid	<i>Glaciecola punicea</i>	<i>Glaciecola pallidula</i>	<i>Alteromonas macleodii</i>
<i>i</i> -13:0	ND	0.3	0.4
13:0	ND	0.1	0.4
<i>i</i> -14:0	ND	0.4	0.2
14:0	1.2	3.6	3.0
14:1 $\omega$ 7c	0.5	3.7	1.1
14:1 $\omega$ 5c	ND	0.2	ND
<i>i</i> -15:0	0.2	ND	0.1
<i>a</i> -15:0	ND	ND	0.5
15:0	1.0	1.6	3.7
15:1 $\omega$ 8c	0.8	3.4	1.3
15:1 $\omega$ 6c	0.4	0.5	0.4
<i>i</i> -16:0	1.8	2.5	2.0
16:0	10.0	9.4	25.0
16:1 $\omega$ 7c	60.7	54.2	34.6
16:1 $\omega$ 5c	0.1	ND	ND
<i>i</i> -17:0	0.5	0.1	0.4
<i>a</i> -17:0	ND	ND	0.6
17:0	0.7	1.3	5.6
17:1 $\omega$ 8c	4.6	5.0	8.0
17:1 $\omega$ 6c	0.4	0.6	0.3
18:0	0.4	0.4	1.0
18:1 $\omega$ 11c	ND	ND	0.6
18:1 $\omega$ 9c	0.2	0.2	0.3
18:1 $\omega$ 7c	16.3	12.6	9.4
19:1	ND	ND	0.2

Abbreviation: ND, not detected.

<sup>a</sup>Measured in weight %.

Data from Bowman et al. (1998c).

## Genus *Idiomarina*

### Introduction

Two strains, KMM 227<sup>T</sup> and KMM 231<sup>T</sup>, were isolated from seawater sampled at a depths of 4000 and 5000 m, respectively, in the northwestern part of the Pacific Ocean. The new isolates were similar in their phenotypic properties to other marine aerobic proteobacteria, including *Alteromonas*, *Pseudoalteromonas* and *Marinomonas*, but they were characterized by several salient differences from known genera: morphological peculiarities, inability to use carbohydrates as the sole source of carbon and energy, and unusual cellular fatty acid composition. On the basis of phenotypic, chemotaxonomic and phylogenetic data, these marine deep-sea bacteria were classified as *Idiomarina abyssalis* and *Idiomarina zobellii* (Ivanova et al., 2000b).

### Phylogeny

Comparative 16S rDNA sequence analysis of *Idiomarina* strains KMM 227 and KMM 231 showed both strains to be similar (sequence similarity 96.9%). They formed a separate monophyletic branch within the  $\gamma$ -subclass of Proteobacteria, and are distantly related to

*Alteromonas*, *Colwellia* and *Pseudoalteromonas*. *Colwellia psychrerythraea* was found to be the nearest relative to *Idiomarina* KMM 227<sup>T</sup> and KMM 231 (90.5 and 90.1% 16S rDNA sequence similarities, respectively; (Ivanova et al., 2000b; Fig. 1).

### Taxonomy

The genus *Idiomarina* was proposed to accommodate strictly aerobic, Gram-negative, psychrotolerant heterotrophic halophilic, marine deep-sea microorganisms with a respiratory type of metabolism. The bacteria require NaCl for growth. The genus includes the type species *Idiomarina abyssalis* KMM 227<sup>T</sup> and *Idiomarina zobellii* KMM 231<sup>T</sup> (Ivanova et al., 2000b). *Idiomarina abyssalis* forms an outer sheath-like structures covering one of the cell poles (Fig. 4a, b). It grows in 0.6–15% NaCl, produces proteinase, and oxidizes cyclodextran, dextran, glycogen, methylpyruvate, monomethylsuccinate, acetate, glycyl-L-glutamic acid, L-proline, glycerol, and glucose-6-phosphate. The G+C content in the DNA is 50 mol%. *Idiomarina zobellii* is characterized by growth in 1–10% NaCl, ability to form long fimbria along with a polar flagellum (Fig. 4c), production of chitinase, and

Table 9. Main phenotypic characteristics differentiating the genus *Glaciecola* from related marine chemoheterotrophic bacteria of the  $\gamma$ -Proteobacteria.

Genus	<i>Glaciecola</i>	<i>Alteromonas</i>	<i>Pseudoalteromonas</i>	<i>Idiomarina</i>	<i>Ferrimonas</i>	<i>Thalassomonas</i>	<i>Colwellia</i> <sup>a</sup>	<i>Shewanella</i>	<i>Moritella</i>
Pigmentation	Pink-red or pale pink	None	None, yellow, red, violet, black, orange, brown, dark green	None or yellowish	None	Green/blue-green diffusible	None, chalk or off-white, red	None, pinkish	None, cream colored
Psychrophilic growth	+	-	V	+	-	-	+	V	+
Growth with 10% NaCl	-	+	V	+	-	-	-	V	-
Facultatively anaerobic	-	-	V	-	+	-	V	+	+
PUFA production	-	-	-	-	-	-	+(DHA)	V(EPA)	+(DHA)
GC content (mol%)	40–46	44–47	37–50	48–50	54	48	35–46	39–55	40–42
Known habitats	Sea ice	Seawater	Sea ice, seawater, fish, bottom sediments, seaweeds, and invertebrates	Deep seawater	Marine sediments	Oysters from Mediterranean coastal water	Sea ice, seawater, benthic sediments, deep seawater, and fish	Sea-ice, deep-sea, fresh waters, sediments, and marine clinical, and food samples	Deep-seawater, sediments, and marine fish

Symbols: +, positive; -, negative; and V, variable between species or strains of the genus.

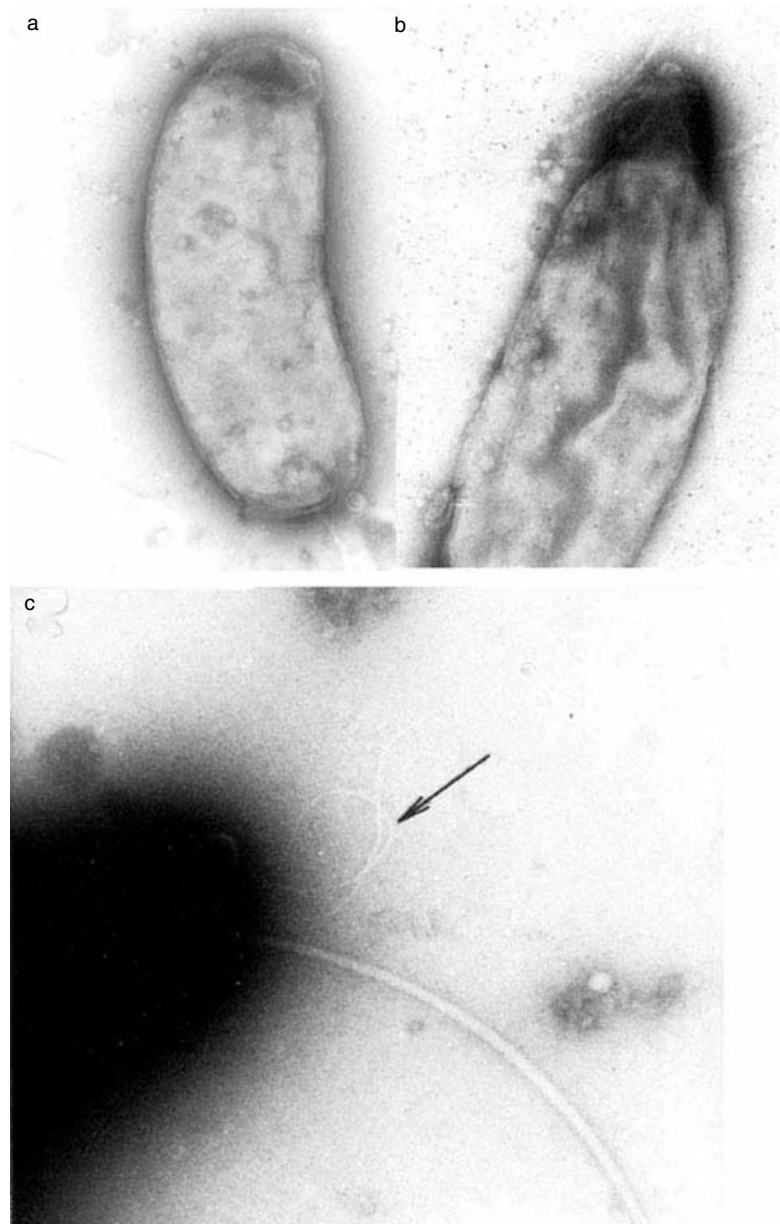
Abbreviations: PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid (20:5 $\omega$ 3); and DHA, docosahexaenoic acid (22:6 $\omega$ 3).

<sup>a</sup>Gas vesicles formation may occur.

Data from Baumann et al. (1972), MacDonell and Colwell (1985), Gauthier et al. (1995), Rossello-Mora et al. (1995), Bowman et al. (1998b, c), Urakawa et al. (1998), Yumoto et al. (1998), Venkateswaran et al. (1999), Benediktsdottir et al. (2000), Ivanova et al. (2000b), and Macian et al. (2001).



Fig. 4. Transmission electronic photos showing morphology of negatively stained cells of *Idiomarina abyssalis* KMM 227<sup>T</sup> (a, b) and *Idiomarina zobellii* KMM 231<sup>T</sup> (c). (a, b) Cells of KMM 227<sup>T</sup> with polar flagella and outer sheath-like structures ballooning over one pole; (c) Cell of KMM 231<sup>T</sup> with fimbria (indicated by arrow) along with a flagellum at one pole.



monomethylsuccinate, glycyl-L-glutamic acid, and L-ornithine utilization. It does not utilize cyclodextran, dextran, glycogen, L-proline, acetate, glycerol, and glucose-6-phosphate and produce proteinase. The G+C content in the DNA is 48 mol%. Comparative characterization of *I. abyssalis* and *I. zobellii* species are shown in Table 10.

#### Habitat

At present, the only known habitat of *Idiomarina* bacteria is deep-sea water (depths of 4000–5000 m), in the Pacific Ocean.

#### Genomic Relatedness

The DNA-DNA reassociation levels between the strains were determined to be as follows: *I. abyssalis* KMM 227<sup>T</sup> and *I. zobellii* KMM 231<sup>T</sup>—27%; *I. abyssalis* KMM 227<sup>T</sup> and other species: *Alteromonas macleodii*—7%; *Vibrio cholerae*—7%; *Aeromonas jandai*—3%; *Marinomonas communis*—7%; *Pseudoalteromonas haloplanktis*—6%; and *Escherichia coli*—3%.

#### Physiology

The genus *Idiomarina* comprises Gram-negative, strictly aerobic, chemoorganotrophic, oxidase-

Table 10. Main phenotypic characteristics differentiating *Idiomarina abyssalis* KMM 227<sup>T</sup> and *Idiomarina zobellii* KMM 231<sup>T</sup>.<sup>a</sup>

Feature	<i>I. abyssalis</i> KMM 227 <sup>T</sup>	<i>I. zobellii</i> KMM 231 <sup>T</sup>
Cell morphology peculiarities	Fimbria	Outer sheath-like structures
Production of chitinase	–	+
Growth at 15% NaCl	+	–
Sensitivity to		
Streptomycin (10µg)	+	–
Erythromycin (15µg)	+	+
Gentamicin (10µg)	+	–

Symbols: +, positive reaction; and –, negative reaction.

<sup>a</sup>Bacteria are positive for the following tests: sodium ion requirement for growth, growth at 30°C, motility by using a single polar flagellum, oxidase, production of lipase, DNase, gelatin liquefaction; are negative for growth at 40°C, indole reaction, nitrate reduction, denitrification, arginine dihydrolyase, and acid production from carbohydrates.

positive, asporogenic, motile by one polar flagellum, rod-shaped, single, and sometimes paired cells of 0.7–0.9 µm in diameter. Bacteria may have fimbria and are able to form external sheath-like structures on the one pole of the cells. They do not accumulate poly-β-hydroxybutyrate as a reserve product and have no arginine dihydrolyase. The bacteria do not need organic growth factors but require seawater or 0.6–15% NaCl for growth. They grow at 4–35°C, with an optimum at 20–25°C, but do not grow at 40°C. Growth occurs at pH 5.5–9.5, with the optimum pH being 7.5–8.5. They produce gelatinases, lipases, and DNases. *Idiomarina abyssalis* produces proteinase. Strains do not hydrolyze starch and agar. The following substrates are utilized: D-arginine, L-tyrosine, L-alanine, alaninamide, L-alanine-glycine, α-ketobutyrate, and α-ketovalerate. Bacteria do not utilize D-arabinose, D-rhamnose, D-mannose, sucrose, maltose, lactose, melibiose, glycerol, mannitol, L-lysine, and L-phenylalanine. Bacteria are sensitive to erythromycin, and tolerant to kanamycin, ampicillin, benzylpenicillin, oleandomycin, lincomycin, tetracycline, oxacillin, vancomycin, and vibriostatic substance O/129. Iso-branched fatty acids with the odd number of carbon atoms predominate (up to 70%), and saturated and monounsaturated fatty acids are detected in cellular fatty acid composition of *Idiomarina* strains.

*Idiomarina* strains are similar in their properties to members of *Alteromonas*, *Pseudoalteromonas*, *Marinomonas*, *Halomonas* and *Oceanospirillum*. Representatives of related *Alteromonas* and *Pseudoalteromonas* genera differ from *Idiomarina* strains by lower DNA G+C content values, ability to utilize a broad spectrum

of carbon compounds, predominance of saturated and monounsaturated cellular fatty acids (Ivanova et al., 2000b). *Marinomonas* species have specific physiological and biochemical properties and a bipolar type of flagellation (Gauthier and Breittmayer, 1992). *Halomonas* species are characterized by tolerance to high concentration of sodium ions, a DNA G+C content of 57–68 mol%, peritrichous flagella and a specific fatty acid pattern (Dobson and Franzmann, 1996). Distinctive features of *Oceanospirillum* are specific cell morphology and phenotypic properties (Krieg, 1984). The main characteristics differentiating *Idiomarina* species from related genera are shown in Table 11.

## Genus *Colwellia*

### Introduction

Abyssal ocean depths (1000–6000 m) are characterized by temperatures <4°C and hydrostatic pressure of 400–600 atm. Bacteria adapted to these conditions can be expected to be psychrophilic, barophilic or barotolerant (ZoBell, 1946; ZoBell and Morita, 1957; Morita, 1975). Deep-sea microorganisms were isolated from many deep-sea samples, including invertebrate amphipods (Yayanos et al., 1979; Yayanos, 1989), intestinal tract of benthic animals (Deming and Colwell, 1982), bottom deposits and seawater (Jannasch et al., 1982; Deming, 1985). Representatives of microbial populations dwelling at ultra-abysal depths from 6000 up to 11,000 m in the world's oceans are the least investigated deep-sea microorganisms.

Barophilic strain BNL-1 were isolated by Deming and coauthors from a seawater sample obtained at a depth of 7410 m in the Puerto Rico Basin of the Atlantic Ocean. Bacteria grew at 2°C and pressure of 740 atm. Phenotypic and genotypic characteristics together with the 5S ribosomal RNA sequence analysis confirmed its difference from the other marine proteobacteria and revealed phylogenetic similarity to both the marine psychrophilic bacterium *Vibrio psychroerythrus* (D'Acoust and Kushner, 1972) isolated from shelf waters, and the deep-sea barophilic *Shewanella benthica* (MacDonell and Colwell, 1985). On the basis of the data obtained, strain BNL-1 was described as *Colwellia hadaliensis*, whereas *Vibrio psychroerythrus* was reclassified as *Colwellia psychroerythrus* comb. nov. (Deming et al., 1988). The epithet “*psychroerythrus*” has been changed to “*psychrerythraea*” (Euzéby, 1998).

Studying the 16S rDNA biodiversity and eco-physiology of bacterial communities associated

Table 11. Characteristics differentiating *Idiomarina* species from related genera.<sup>a</sup>

Characteristic	Strain/genus							
	1	2	3	4	5	6	7	8
Cell diameter (µm)	0.7–0.9	0.7–0.9	0.7–1.0	0.5–1.0	0.6–1.1	0.7–1.5	0.3–1.4	0.2–1.5
Flagella arrangement:								
Polar	+	+	+	+	+	–	+	+
Bipolar	–	–	–	–	–	+	+	–
Lateral	–	–	–	–	+	–	–	+
Organelles	–	Fimbria	–	–	Filaments	–	–	–
Metabolism	Aerobic	Aerobic	Aerobic	Facultatively anaerobic	Aerobic	Aerobic	Aerobic	Aerobic or facultatively anaerobic
Growth occurs in NaCl (%)	0.6–15	1–10	1–10	>2.5	0–30	1–6	0.5–10	1–12–15
At 4°C	+	+	–	+	V	–	–	V
Presence of:								
Poly-β-hydroxybutyrate	–	–	–	ND	+	–	+	–
Gelatinase, lipase	+	+	+	+	–	–	–	+
Chitinase	–	+	V	+	–	–	–	V
Acid production from sugars	–	–	+	+	+	+	–	V
Utilization of:								
D-glucose	–	–	+	+	+	+	–	V
D-fructose	–	–	+	–	+	+	–	V
Mannitol	–	–	V	–	+	+	–	V
Sucrose	–	–	+	–	+	–	–	V
Maltose	–	–	+	–	+	V	–	V
Lactose	–	–	+	–	+	–	–	V
Major fatty acid	Iso-branched	Iso-branched	Straight	Straight	Straight	ND	Straight	Straight
DNA GC content (mol%)	50	48	44–47	35–46	52–68	45–50	42–51	37–50

Symbols: +, trait present; –, trait absent; V, variable between strains; and ND, not determined.

<sup>a</sup>Strains or genera: 1, *I. abyssalis* KMM 227<sup>T</sup>; 2, *I. zobellii* KMM 231<sup>T</sup>; 3, *Alteromonas*; 4, *Colwellia*; 5, *Halomonas*; 6, *Marinomonas*; 7, *Oceanospirillum*; and 8, *Pseudoalteromonas*.

Data obtained from D'Aoust and Kushner (1972), Baumann et al. (1984a, b), Krieg (1984), Vreeland et al. (1980), Deming et al. (1988), Bowman et al. (1998b), and Ivanova et al. (2000b).

with diatom algae assemblages of the Antarctic sea ice, Bowman and coauthors identified eight isolates similar to *Colwellia* strains. The isolates were psychrophilic and facultatively anaerobic, nonpigmented bacteria, growing in seawater, and able to synthesize ω3 polyunsaturated fatty acid docosahexaenoic acid, DHA, (0.7–8.0% of the total fatty acid amount). DNA-DNA hybridization similarities revealed the new Antarctic isolates form five distinct genospecies including *Colwellia psychrerythraea*. Thus, four new species of Antarctic DHA-producing bacteria have been described, i.e., *Colwellia demingiae*, *Colwellia psychrotropica*, *Colwellia rossensis* and *Colwellia hornerae* (Bowman et al., 1998b).

Phylogenetic, cultural, chemotaxonomic and enzymological properties (Takada et al., 1979) on the psychrophilic bacterium *Vibrio* sp. ABE-1, isolated from marine environment, allowed this strain to be identified as a new species of *Colwellia*, *C. maris* (Yumoto et al., 1998).

## Phylogeny

16S rDNA sequence analysis showed 95.2–100% similarity between *Colwellia demingiae*, *Colwellia psychrotropica*, *Colwellia rossensis*, *Colwellia hornerae* and *Colwellia psychrerythraea* (96–97% sequence similarity). *Alteromonas macleodii*, *Glaciecola* and *Pseudoalteromonas* species were moderately related to *Colwellia* species (87–89% similarity; Bowman et al., 1998b). The 16S rDNA sequence analysis of *Colwellia maris* ABE-1<sup>T</sup> revealed 92.9% similarity to *C. psychrerythraea* and 97.8% similarity to *C. rossensis*, while only low similarities were found with strains of *Pseudoalteromonas*, *Alteromonas*, *Shewanella*, *Photobacterium*, *Vibrio*, *Moritella*, *Escherichia* and *Aeromonas* (less than 90%).

## Taxonomy

The genus *Colwellia* was proposed by Deming et al. (1988) for curved or straight rod-shaped

(0.5–1.0  $\mu\text{m}$  in diameter and 2.5–5.0  $\mu\text{m}$  in length), Gram-negative, asporogenic, motile by polar flagella, catalase- and oxidase-positive, chemoorganotrophic, facultative anaerobic microorganisms, associated with deep marine environments. All strains are able to synthesize docosahexaenoic acid, and the DNA G+C content is 40–46 mol%. The type species *Colwellia psychrerythraea* is red-pigmented and psychrophilic. Currently the genus *Colwellia* comprises seven species, including *Colwellia psychrerythraea*, *Colwellia hadaliensis* (Deming et al., 1988), *Colwellia demingiae*, *Colwellia rossensis*, *Colwellia hornerae*, *Colwellia psychrotropica* (Bowman et al., 1998b) and *Colwellia maris* (Yumoto et al., 1998).

### Habitat

*Colwellia* strains have been isolated from organic materials and coastal marine water samples collected offshore from the United States (DeLong et al., 1993; Suzuki et al., 1997) and Marianas Trench (DeLong and Yayanos, 1986); Antarctic meromictic lake, Antarctic sea ice and water samples (Gosink and Staley, 1995; Bowman et al., 1998b); and cold coastal waters near Hokkaido, the Sea of Japan (Yumoto et al., 1998). *Colwellia* species are common inhabitants of the cold sea environments and have not been isolated from any other environments.

### Physiology

Members of *Colwellia* are psychrophilic with an growth temperature at  $<15^{\circ}\text{C}$ , requiring sodium and magnesium ions for growth. Bacteria may be barophilic; *C. hadaliensis* is obligately barophilic. *Colwellia rossensis* forms intracellular gas vesicles. Most strains are capable of synthesizing  $\omega 3$  polyunsaturated fatty acid docosahexaenoic acid (22:6 $\omega 3$ ; 0.7–8.0% of total fatty acids). All strains reduce nitrate to nitrite and degrade chitin and starch under aerobic conditions. Carbohydrate oxidation, fermentation of *N*-acetylglucosamine, chitin and D-glucose, and hydrolysis of other substrates are varied between *Colwellia* strains and species. The main phenotypic properties for differentiation of Antarctic *Colwellia* species and *Colwellia psychrerythraea* are listed in Table 12.

*Colwellia maris* has characteristic features and can be differentiated from other *Colwellia* species in its inability to ferment glucose and hydrolyze chitin, its gelatinase and DNase production, and unique synthesis of 9-*trans*-hexadecenoic acid (Table 13). Yumoto and coauthors reported that *C. psychrerythraea* did not generally produce acid from D-glucose under aerobic or anaerobic conditions for as long as 1 month, but acid was occasionally produced under aerobic conditions. The definition of *Colwellia* should

thus be amended with respect to D-glucose metabolism (Yumoto et al., 1998).

### Genomic Relatedness

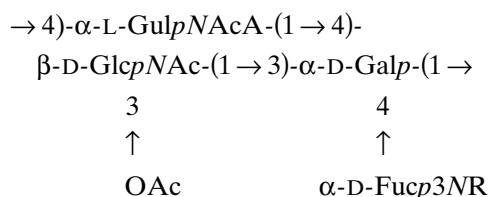
The DNA G+C content of the most *Colwellia* species range from 35 to 42 mol% (Table 13). A higher DNA G+C content of 45.7 mol% has been noted for *C. hadaliensis*. The DNA-DNA reassociation levels between *Colwellia* species were found to be as follows: 35–40% reassociation between *C. psychroerythraea* and *C. demingiae*; 12–24% between *C. psychroerythraea* and *C. rossensis*, *C. hornerae* and *C. psychrotropica* (Bowman et al., 1998b); and 22% between *C. psychroerythraea* and *C. maris* (Yumoto et al., 1998).

## Chemotaxonomic Characterization

### Carbohydrate-containing Biopolymers of *Pseudoalteromonas* Species

Structural investigations of polysaccharides (PSs) of several *Pseudoalteromonas* strains established a primary structure of repeating units of PSs in *P. nigrifaciens*, *Pseudoalteromonas* spp., and *P. elyakovii* (Gorshkova et al., 1993; Gorshkova et al., 1997; Gorshkova et al., 1998a; Gorshkova et al., 1998b; Nazarenko et al., 1993a; Nazarenko et al., 1993b; Nazarenko et al., 1999). The capsulated representatives of *Pseudoalteromonas* were described by Ivanova et al. (Ivanova, 1994a; Ivanova, 1996a). Capsular polysaccharides contained fucose, rhamnose, mannose, glucose, galactose, galactosamine, glucosamine, heptose, 3-deoxy-2-octulosonic acid and uronic acid. Chemical and physicochemical methods (mainly high-resolution NMR spectroscopy) revealed that capsular and cellular antigenic PSs had an identical structure (with the exception of those in *P. elyakovii*).

*Pseudoalteromonas nigrifaciens* strains, isolated from mollusks inhabiting the Sea of Japan, contain an unusual capsular PS structure composed of tetrasaccharide repeating units containing D-galactose, 3-*O*-acetyl-2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-L-guluronic acid and 3,6-dideoxy-3-(4-hydroxybutyramido)-D-galactose (Fuc3NR) residues. The structure of the repeating unit is as follows:



where R is -CO-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-OH

Table 12. Phenotypic characteristics of *Colwellia psychroerythraea* and Antarctic *Colwellia* species.<sup>a</sup>

Characteristic	<i>Colwellia psychroerythraea</i>	<i>Colwellia demingiae</i>	<i>Colwellia rossensis</i>	<i>Colwellia hornerae</i>	<i>Colwellia psychrotropica</i>
Prodigiosin-like pigments	V+	-	-	-	-
Growth at 25°C	-	-	-	-	+
Gas vesicles	-	-	+	-	-
Barophilic growth	-	-	-	-	-
Toleration of 200% sea salts	V-	-	-	+	+
Susceptibility to vibriostatic agent O/129 (100µgml <sup>-1</sup> )	V-	-	-	+	-
Hydrolysis of					
Urea	V+	-	+	-	+
Uric acid	-	-	-	-	+
Tween 80	+	-	NG	+	+
Casein	+	+	-	+	+
Gelatin	V+	-	-	-	-
Esculin	+	+	NG	+	-
Chitin	+F	+F	+F	-	+F
Starch	+	+	+	+	-
Tyrosine	V-W	-	-	-	+ <sup>b</sup>
Production of acid from					
L-Rhamnose	-	-	-	+F	-
D-Glucose	+F	-	+F	-	-
D-Galactose	-	-	+	-	-
N-Acetylglucosamine	+F	+F	+F	-	+F
Cellobiose	V+W	-	-	-	-
Maltose	+F	-	-	-	-
Glycerol	-	-	+	+	-
Utilization of					
D-Glucose	V+	-	+	-	-
L-Arabinose, D-fructose, and D-gluconate	-	-	+	-	-
N-Acetylglucosamine	+	+	+	-	+
Glycerol	-	-	+	+	-
Glycogen	+	V-	+	-	-
Propionate	V-	+	-	+	-
Isobutyrate	-	V+	-	-	+
Valerate, caproate, γ-aminobutyrate	-	+	+	+	+
Heptanoate	-	V+	-	+	-
Caprylate	-	V+	-	-	-
Malonate	-	V+	+	-	-
Glutarate	-	+	-	+	-
Azelate	-	V+	-	+	-
Citrate	-	+	+	+	-
2-Oxoglutarate	-	-	-	-	+
3-Hydroxybutyrate	+	-	+	-	+
L-Malate	-	-	+	-	+
DL-Lactate	V+	+	-	+	+
Oxaloacetate	+	+	-	+	+
L-Alanine, and L-aspartate	-	V+	+	-	+
L-Asparagine	-	+	+	-	+
L-Phenylalanine	-	V+	-	-	-
Hydroxy-L-proline	-	V+	-	+	-
L-Serine	-	+	-	-	-

Symbols: +, all strains positive for test; V+, variable reactions for test, type strain is positive; V-, variable reactions for test, type strain is negative; -, all strains negative for test; W, weak or delayed reaction; F, acid (but not gas) was formed from these substrates in Leifson oxidation/fermentation medium; and NG, no growth occurred on test medium.

<sup>a</sup>The following tests were positive for all strains: catalase, cytochrome-*c* oxidase, nitrate reduction, alkaline phosphatase, growth at 0–15°C and utilization of acetate, butyrate, succinate, fumarate, pyruvate, L-glutamate and L-proline as sole sources of carbon and energy. The following tests were all negative: Fe(III) reduction; trimethylamine N-oxide (TMAO) reduction, growth on 0–25% sea salts or ≥400% sea salts, hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG), DNA and dextran, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activity, production of indole from L-tryptophan, H<sub>2</sub>S production, production of acid from arabinose, xylose, melibiose, fructose, lactose, mannitol, sorbitol, inositol, mannose, mannose, sucrose and trehalose, and utilization of D-galactose, D-xylose, cellobiose, lactose, maltose, D-melibiose, trehalose, sucrose, L-raffinose, D-adonitol, D-arabitol, *m*-inositol, D-mannitol, D-sorbitol, D-gluconate, saccharate, *a*-glycerophosphate, isovalerate, nonanoate, adipate, pimelate, aconitate, L-threonine, L-valine and putrescine.

<sup>b</sup>Brown diffusible pigment formed during L-tyrosine hydrolysis.

Data from Bowman et al. (1998b).

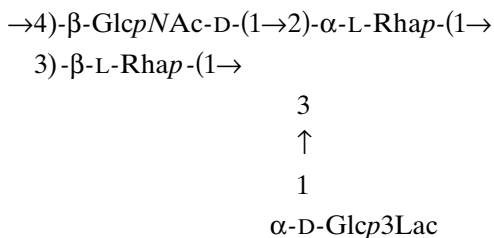
Table 13. Phenotypic characteristics differentiating *Colwellia* species.<sup>a</sup>

Characteristic	<i>Colwellia psychroerythraea</i>	<i>Colwellia hadaliensis</i>	<i>Colwellia demingiae</i>	<i>Colwellia rossensis</i>	<i>Colwellia hornerae</i>	<i>Colwellia psychrotropica</i>	<i>Colwellia maris</i>
Prodigiosin-like pigments	V+	-	-	-	-	-	-
Growth at 25°C	-	-	-	-	-	+	-
Gas vesicles	-	-	-	+	-	-	-
Barophilic growth	-	+	-	-	-	-	-
Na <sup>+</sup> or sea salts required for growth	+	ND	+	+	+	+	+
Susceptibility to vibriostatic agent O/129 (100 µg ml <sup>-1</sup> )	V-	ND	-	-	+	-	+
Acid production acid from							
Glucose	+	+	-	+	-	-	+
Maltose	+	ND	-	-	-	-	-
L-Arabinose, melibiose, D-fructose, and D-mannose	-	ND	-	-	-	-	-
Hydrolysis of							
Tween 80	+	ND	-	NG	+	+	+
Gelatin	V+	ND	-	-	-	-	+
Starch	+	ND	+	+	+	-	-
Chitin	+F	+	+F	+F	-	+F	-
DNA	-	ND	-	-	-	-	+
Utilization of							
D-Glucose	V+	ND	-	+	-	-	+
L-Arabinose, and D-fructose	-	ND	-	+	-	-	-
Maltose, lactose, D-xylose, melibiose, and raffinose	-	ND	-	-	-	-	-
Glycerol	+	ND	+	+	-	+	-
DNA G+C (mol%)	35-38	45.7	37	38	39	42	39.4

Symbols and Abbreviations: +, all strains positive for test; V+, variable reactions, type strain is positive; V-, variable reactions for test, type strain is negative; -, all strains negative; F, acid (but not gas) was formed from the substrate in Leifson O/F medium; ND, not detected; and NG, no growth occurred on test medium.

Data were obtained from Deming et al. (1988), Bowman et al. (1998b), and Yumoto et al. (1998).

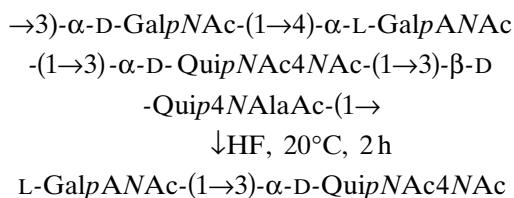
The polysaccharide of *P. nigrifaciens* KMM 156 is composed of branched tetrasaccharide repeating units containing two L-rhamnose residues, one 2-acetamido-2-deoxy-D-glucose residue and a 3-O-[(R)-1-carboxyethyl]-D-glucose residue (Glc3Lac) and had the following structure:



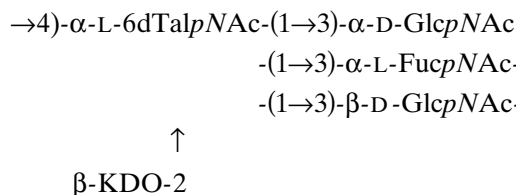
where  $\alpha - \text{D} - \text{Glc}p3\text{Lac}$  is 3-O-[(R)-1-carboxyethyl]-D-glucose. This unusual sugar is

the deamino-analog of muramic acid—one of the components of the peptidoglycan of the bacterial cell wall. Besides, this acidic sugar was also identified as a component of extracellular PS of *Pseudomonas fragi* and the O-antigenic PS from *Proteus vulgaris*

Cellular antigenic PS of *P. nigrifaciens* KMM 155 is composed of tetrasaccharide repeating units containing only amino sugars: 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-L-galacturonic acid and two D-bacillosamine, (3,6-dideoxy-D-glucose) residues, one of which is N-acetylated at the position 4 and the second one is N-acylated by a of N-acetylalanine residue. As a result of hydrogen fluoride (HF) solvolysis following gel-permeation chromatography, the disaccharide was isolated:



According to data of the carbohydrate analysis and NMR data, the antigenic PS of *P. nigrifaciens* IAM 3010<sup>T</sup> contained two D-glucosamine, L-fucosamine, 6-deoxytalosamine (pneumosamine) and 3-deoxy-2-octulosonic acid (KDO) residues. The polysaccharide had the following structure:

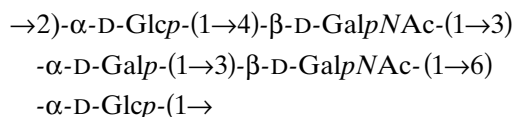


where 6dTalpNAc is 2-acetamido-2,6-dideoxy-L-talopyranose (pneumosamine)

Antigenic PS of marine bacteria of *Pseudoalteromonas* are defined as either linear or branched glycosaminoglycans which contain rare and unusual *N*-acylamino- and acidic sugars such as: 3-*O*-[(*R*)-1-carboxyethyl]-D-glucose (glucolactylic acid; *P. nigrifaciens* KMM 156); 3,6-dideoxy-3-(4-hydroxybutyramido)-D-galactose and 2-acetamido-2-deoxy-L-guluronic acid (*P. nigrifaciens* KMM 158); di-*N*-acetyl-D-bacillosamine and 4-(*N*-acetyl-D-alanyl)amino-4,6-dideoxy-D-glucose; *P. nigrifaciens* KMM 155); and 2-acetamido-2,6-dideoxy-L-talose (*N*-acetyl-pneumosamine; *P. nigrifaciens* IAM 3010<sup>T</sup>).

The O-specific PS of *Pseudoalteromonas elyakovii* KMM 162<sup>T</sup> was studied in detail. The chain is a peripheral part of lipopolysaccharide (LPS) and may be released from the bacterial cell into the surrounding space at a distance of 10–12 nm. Usually, the O-antigen is a regular heteropolysaccharide, occurring in some cases as a homopolysaccharide, linear or branched, built up of oligosaccharide repeating units (from di- to hexasaccharide, less commonly from hepta- and even octasaccharide). Components of PSs may contain various monosaccharides and non-carbohydrate substitutes. The polysaccharide chain of LPS may be neutral or acidic, but as a rule, the concentration of a negative charge does not exceed one acidic group per three monosaccharide residues. The structure of O-specific PS from *P. elyakovii* 162<sup>T</sup> was established on the basis of the carbohydrate analysis data, methylation study, and <sup>13</sup>C-NMR-spectroscopy study. This PS contains D-glucose, D-galactose, and 2-

acetamido-2-deoxy-D-galactose residues in the ratio of 2:1:2. The structure of the O-specific PS is as follows:



However, capsular PS of this strain has a different sugar composition (R. P. Gorshkova and E. L. Nazarenko, personal communication).

The O-specific PS of *Pseudoalteromonas* sp. KMM 634 was found to contain D-glucuronic acid, 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid, 2,3-diacetamido-2,3-dideoxy-D-mannuronoyl-L-alanine, and 2-acetamido-2,4,6-trideoxy-4-[(*S*)-3-hydroxybutyramido]-D-glucose (Komandrova et al., 2000).

A sulfated O-specific PS containing D-mannose, L-rhamnose, and the sulfate group was obtained by mild acid hydrolysis of lipopolysaccharide (S-form) of “*Pseudoalteromonas marinoglutinosa*” KMM 232. This is the first report of a sulfated O-specific polysaccharide from a Gram-negative bacterium (Komandrova et al., 1998). An O-specific PS was obtained by mild degradation of the lipopolysaccharide of the marine bacterium “*Alteromonas marinoglutinosa*” NCIMB 1770 and found to contain D-galactose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-mannosamine residues (Komandrova et al., 2001).

An acidic polysaccharide was obtained from the lipopolysaccharide of *Pseudoalteromonas distincta* KMM 638<sup>T</sup>, isolated from a marine sponge, and found to contain D-glucuronic acid (D-GlcA), 2-acetamido-2-deoxy-D-galactose (D-GalNAc), 2-acetamido-2,6-dideoxy-D-glucose (D-QuipNAc), and two unusual acidic amino sugars: 2-acetamido-2-deoxy-D-galacturonic acid (D-GalNAcA) and 5-acetamido-3,5,7,9-tetradeoxy-7-formamido-L-glycero-L-mannonulosonic acid (Pse5Ac7Fo, a derivative of pseudaminic acid; Muldoon et al., 2001).

Thus, PSs of marine aerobic proteobacteria incorporate rare and unusual acidic and *N*-acylamino sugars as well as noncarbohydrate substitutes, and they differ by a structural diversification even among strains of the same species. These variations may be a reflection of different eukaryotic hosts (mollusks, sponges and ascidians) and different ecological niches.

Some *Alteromonas*-*Pseudoalteromonas*-like producers of exogenous polysaccharides (exoPSs) and the role of these extracellular polysaccharides in heavy metal binding (Vincent et al., 1994) and bacterial cell attachment have been reported (Holmström and Kjelleberg, 1999). “*Alteromonas macleodii* subsp. *fijiensis*”

(Ragueneas et al., 1996), and “*A. infernus*” (Ragueneas et al., 1997) produce acidic exoPSs. During periodical cultivation in a liquid medium with glucose (30 g/liter) cells of “*A. infernus*” from stationary phase excreted an unusual water-soluble exoPS, which contained glucose, galactose, galacturonic and glucuronic acids (Ragueneas et al., 1997).

### Fatty Acid and Phospholipid Composition

Fatty acid (FA) and phospholipid (PL) compositions are widely used in identification. Owing to the high metabolic similarity of marine aerobic heterotrophs, application of chemotaxonomy should be useful for rapid and accurate identification at least up to the genus level of environmental isolates.

Fatty acid composition of type strains and environmental isolates are available for members of *Pseudoalteromonas* (Table 14), *Alteromonas* and *Marinomonas* (Matsui et al., 1991; Svetashev et al., 1995; Ivanova et al., 2000c). Among 30 fatty acids with 11 to 19 carbon atoms detected in alteromonads and pseudoalteromonads and the 16 fatty acids found in marinomonads, acids C16:1 (n-7), C16:0, C17:1 (n-8), and C18:1 (n-7) were predominant, whereas C14:0, C15:0, C15:1 (n-8), and C17:0 were minor components. The absence of cyclopropanoic FA and hydroxy FA are characteristic features of all strains studied. *Marinomonas* species had the simplest FA composition with only three major components (C16:0, C16:1 [n-7], and C18:1 [n-7]), which generally accounted for more than 90% of the total fatty acids. Branched-chain fatty acids were not detected. Specifically, the amount of C18:1 (n-7) reached 44.4–52.8%. It should be noted that two strains of *Marinomonas*, *M. vaga* and *M. communis*, showed highly similar fatty acid profiles. *Marinomonas mediterranea* (Solano and Sanchez-Amat, 1999) showed differences in C16:0 and C18:1(n-7), while still showing the genus-specific fatty acid profile (Table 15).

Overall, PLs accounted for from 55% (mainly for pigmented species) to 85% of the lipid extracts. The thin layer chromatography (TLC) studies revealed that phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG) were the major phospholipids of agar cultures of *Pseudoalteromonas*, *Alteromonas* and *Marinomonas*. The proportion of these PL components had a characteristic genus-specific pattern (Table 16). The amounts of PE ranged from 56.9 to 67.7% for *Alteromonas* spp., from 78.1 to 87% for *Pseudoalteromonas* spp., and from 60.7 to 68.8% for *Marinomonas* spp., while the amounts of PG ranged from 32.3 to 43.1%, from 13.0 to 21.9%, and from 31.2 to 35.1%, respectively. A

glycolipid was detected in all lipid extracts of agar cultures. Neither diphosphatidyl glycerol (DPG) nor glycopospholipids were found. Differences in the PL compositions of the same strains grown in liquid and on solid media have been detected (Frolova et al., 2000; Ivanova et al., 2000c).

## Family Alteromonadaceae

Ivanova and Mikhailov proposed a new family, Alteromonadaceae, comprising *Alteromonas* and related genera (Ivanova and Mikhailov, 2001a). Phylogenetic analyses have shown that the genera of *Alteromonas*, *Pseudoalteromonas* and *Idiomarina* represent a distinct cluster within the  $\gamma$ -subclass of the class Proteobacteria with a 16S rDNA similarity level of 96.9%, which corresponds to 45 differences per 1462 sequenced nucleotides (Fig. 1). Phenotypic and genotypic characteristics of the genera of the family Alteromonadaceae are presented in Tables 15, 16 and 17. The genus *Glaciecola* described by Bowman et al. (1998c) is phylogenetically closely related to *Alteromonas macleodii* and could be regarded as a member of this family. Thus, the genera of *Alteromonas*, *Pseudoalteromonas*, *Glaciecola*, *Idiomarina* and *Colwellia* comprise a monophyletic evolutionary line at a 95–100% confidence level. The 16S rDNA similarity values obtained for these genera are 90% or higher. However, R. Christen and E. Ivanova propose to create several new families, including Pseudoalteromonadaceae (genus *Pseudoalteromonas*), Colwelliaceae (genera *Colwellia*, *Idiomarina* and *Thalassomonas*) and an emended Alteromonadaceae (genera *Alteromonas* and *Glaciecola*; R. Christen and E. Ivanova, in preparation).

## Isolation, Cultivation, and Identification

### Isolation

The isolation of *Alteromonas*, *Pseudoalteromonas*, *Idiomarina* and certain *Colwellia* species is based on common microbiological techniques for autochthonous marine, heterotrophic aerobic or facultatively anaerobic bacteria. See, e.g., ZoBell (1946).

Many techniques described in this chapter originate from investigations of Mikhael Doudoroff and coauthors (Stanier et al., 1966), Linda and Paul Baumann and coauthors (Baumann et al., 1971; Baumann et al., 1972; Baumann and Baumann, 1981), and from Michel Gauthier and Violette Breittmayer (Gauthier and Breittmayer, 1992).



Table 14. Fatty acid composition<sup>a</sup> of *Pseudodalteromonas* type strains.<sup>b</sup>

Fatty acid	<i>P. haloplanktis</i> ATCC 14393 <sup>T</sup>	<i>P. tetraodonis</i> IAM 14160 <sup>T</sup>	<i>P. atlantica</i> IAM 12927 <sup>T</sup>	<i>P. carrageenovora</i> IAM 12662 <sup>T</sup>	<i>P. antarctica</i> CECT 4664 <sup>T</sup>	<i>P. undina</i> IAM 2992 <sup>T</sup>	<i>P. espejiana</i> ATCC 19659 <sup>T</sup>	<i>P. elyakovii</i> ATCC 700519 <sup>T</sup>
12:0	2.00	1.50	1.90	0.90	1.48	1.52	1.90	1.00
12:1	0.30	0.30	0.30	0.20	0.66	0.68	0.63	0.10
<i>i</i> -13:0	0.10	0.00	0.10	0.00	0.07	1.67	U	0.00
13:1	0.30	0.50	0.30	0.20	0.38	0.40	0.30	0.20
14:0	2.00	2.00	3.90	0.90	0.75	1.30	0.83	1.40
14:1(n-7)	0.60	1.20	1.40	0.50	0.57	0.93	0.41	0.50
15:0	3.30	8.00	2.20	5.10	4.25	2.88	4.19	3.60
15:1(n-8)	2.30	6.30	1.50	2.10	2.54	3.45	1.59	1.40
<i>i</i> -16:0	0.20	1.40	0.80	0.70	2.36	2.29	0.70	1.30
16:0	30.10	18.20	29.10	22.30	20.58	13.93	22.73	29.00
16:1(n-7)	40.50	35.00	47.20	45.00	40.86	44.63	37.84	46.90
<i>a</i> -17:0	0.00	0.60	0.40	0.20	1.29	2.11	0.40	0.90
12:0-3OH	1.90	0.90	1.60	1.00	0.63	0.79	0.41	1.00
17:0	3.90	5.50	0.50	3.40	3.21	1.41	4.75	1.60
17:1(n-8)	6.00	10.70	1.60	8.80	8.65	6.05	10.05	4.80
17:1(n-6)	0.20	0.80	0.00	0.60	0.49	0.47	0.64	0.30
18:0	1.90	0.60	0.90	0.60	1.45	0.57	1.13	0.60
18:1(n-9)	0.20	0.20	0.40	0.20	0.40	0.19	0.20	0.40
18:1(n-7)	2.40	2.50	3.20	4.40	5.90	6.28	8.30	2.80

Table 14. Continued

Fatty acid	<i>P. rubra</i> ATCC 29570 <sup>T</sup>	<i>P. luteoviolacea</i> NCIMB 1893 <sup>T</sup>	<i>P. aurantia</i> NCIMB 2033	<i>P. tunicata</i> CCUG 26757 <sup>T</sup>	<i>P. citrea</i> ATCC 29719 <sup>T</sup>	<i>P. piscicida</i> NCIMB 645 <sup>T</sup>	<i>P. nigrifaciens</i> IAM 13010 <sup>T</sup>	<i>P. bacteriolytica</i> E8R <sup>T</sup>
12:0	U	U	U	0.60	0.79	U	0.80	0.70
12:1	0.54	0.47	0.53	3.00	0.60	0.97	0.00	0.10
<i>i</i> -13:0	0.00	0.26	0.00	U	0.00	0.00	0.00	0.40
13:1	0.48	1.01	0.50	0.30	0.15	0.14	0.00	0.90
14:0	1.60	3.94	0.99	1.80	3.10	2.64	1.70	8.40
14:1(n-7)	0.46	0.83	0.24	0.80	0.99	0.67	0.40	0.60
15:0	3.78	2.62	2.68	2.10	2.98	2.17	2.60	0.20
15:1(n-8)	1.68	1.37	2.35	1.10	3.80	1.36	0.60	U
<i>i</i> 16:0	1.71	1.14	1.96	0.50	1.12	1.23	0.20	U
16:0	16.5	17.13	17.58	17.20	21.24	25.69	33.80	27.80
16:1(n-7)	34.44	33.71	29.56	46.30	41.20	39.88	47.00	42.90
<i>a</i> -17:0	0.00	0.00	0.16	0.50	0.31	0.00	0.10	0.60
12:0-3OH	0.22	0.17	0.16	U	1.09	0.17	0.10	2.80
17:0	3.58	3.25	6.72	1.60	1.57	2.25	1.90	U
17:1(n-8)	14.23	9.4	18.95	5.90	6.44	6.15	5.00	0.20
17:1(n-6)	0.59	1.14	1.31	0.50	0.55	0.40	0.20	U
18:0	0.61	0.84	0.65	1.00	0.52	1.79	0.80	0.70
18:1(n-9)	0.48	0.37	0.37	0.40	0.24	0.28	0.50	1.90
18:1(n-7)	11.19	16.03	6.85	14.1	7.09	8.74	1.70	2.40

Abbreviation: <sup>T</sup>, type strain; U, undetectable.

<sup>a</sup>Measured in weight %.

<sup>b</sup>The following fatty acids were determined at levels of <1.0%: 13:0, *i*-14:0, *i*-15:0, *a*-15:0, 15:1(n-6), 11:0-3OH, 16:1(n-5), *i*-17:0, *i*-18:0, 18:1(n-11), and 19:1.

Table 15. Cellular fatty acids of *Alteromonas*, *Pseudoalteromonas*, *Glaciecola*, *Idiomarina*, *Colwellia*, *Thalassomonas* and *Marinomonas*.

Fatty acid	<i>Alteromonas macleodii</i>						
	ATCC 27126 <sup>T</sup>	<i>Pseudoalteromonas</i>	<i>Idiomarina</i>	<i>Colwellia</i>	<i>Glaciecola</i>	<i>Thalassomonas</i>	<i>Marinomonas</i>
11:0-3OH	0.2	0–0.4	U	U	ND	3.0–4.2	U
12:0-3OH	0.4	0.4–1.97	U	U	ND	5.0–6.0	U
12:0	1.2	0.6–2.0	0.2	U	ND	1.6–2.0	0.2–1.8
11:0	U	U	0.1	U	ND	0.9–1.1	U
12:1	U	0–1.1	U	U	ND	ND	0.8–2.7
<i>i</i> -13:0	0.2	0.1–0.2	1.0	U	0.3	ND	U
13:1	U	0.1–0.5	U	U	ND	ND	U
<i>i</i> -14:0	0.2	0–0.2	0.1	U	0.4	ND	U
14:0	3.5	1.1–3.9	0.6	0.8–8.0	1.2–3.6	2.3–3.0	2.7–4.7
14:1(n-7)	0.9	0.4–1.4	0.1–0.2	2.0–9.3	0.5–3.7	ND	0.2
<i>i</i> -15:0	U	0.1–0.2	33.7–40.6	U	0.2	<1	U
<i>a</i> -15:0	0.5	0.1–0.8	0.6	U	ND	ND	0.7–1.0
15:0	3.2	2.2–8.5	U	1.7–14.3	1.0–1.6	ND	0.1–0.2
15:1(n-6)	0.2	0–0.8	0.1–0.3	0–1.1	0.4–0.5	0.4–1.2	U
15:1(n-8)	2.3	0.6–7.3	1.1–1.3	1.9–20.3	0.8–3.4	4.5–5.8	U
<i>i</i> -16:0	0.8	0.2–2.12	U	0–10.3	1.8–2.5	<1	U
16:0	23.8	14.0–33.8	4.6–6.3	U	9.4–10.0	11.2–13.7	14.0–20.7
16:1(n-5)	U	0–0.3	U	U	0.1	ND	0.2
16:1(n-7)	37.1	35.0–49.1	7.0–8.3	15.4–43.4	54.2–60.7	21.2–28.4 <sup>b</sup>	23.1–23.7
16:1(n-9)	U	U	0.5–0.6	1.8–11.8	ND	ND	U
<i>i</i> -17:0	U	U	11.9–12.5	U	0.1–0.5	<1	0.1
<i>a</i> -17:0	0.6	0–1.0	0.2	U	ND	ND	0.3
17:0	4.5	0.5–5.5	0.5–0.6	0–2.5	0.7–1.3	3.1–4.7	0.1–0.2
17:1(n-6)	0.3	0–0.8	1.5–3.4	0–1.9	0.4–0.6	<1	U
17:1(n-8)	7.0	1.6–12.3	0.8–1.1	0–5.6	4.6–5.0	ND	0.1–0.3
<i>i</i> -18:0	0.4	0–0.2	U	U	ND	ND	U
18:0	1.0	0.5–1.9	0.8–1.8	0.1–2.4	0.4	<1	1.4–1.8
18:1(n-7)	9.4	1.3–5.7	5.9–6.7	0.3–4.2	12.6–16.3	3.2–6.1	44.4–53.0
18:1(n-9)	0.3	0.2–0.6	0.9–1.4	0–1.4	0.2	<1	U
18:1(n-11)	0.6	0–0.8	U	U	ND	ND	0.1–0.2
19:1	0.2	0.03	U	U	ND	ND	U
20:5(n-3)	U	U	U	0–1.5	ND	ND	U
22:6(n-3)	U	U	U	1.7–4.1	ND	ND	U

Abbreviations: <sup>T</sup>, type strain; U, undetectable; and ND, not determined.

<sup>a</sup>The fatty acid 13:0 was determined at levels of <1.0%.

<sup>b</sup>Summed value of 16:1(n-7) and *i*-15:2-OH mixture.

Data from Bowman et al. (1998b, c), Ivanova and Mikhailov (2001a), and Macian et al. (2001).

Table 16. Phospholipid composition of *Pseudoalteromonas*, *Alteromonas*, and *Marinomonas*.

Feature	<i>Alteromonas</i>	<i>Pseudoalteromonas</i>	<i>Idiomarina</i>	<i>Colwellia</i>	<i>Glaciecola</i>	<i>Thalassomonas</i>
Flagellation					ND	
Polar	+	+	+	+		+
Lateral	–	+	–	–		–
Other outer organelles	–	–	Fimbria or sheath-like structure	–	Filaments	–
Pigmentation	–	V	–	V	+	+
Metabolism	Aerobic	Aerobic or facultatively anaerobic	Aerobic	Facultatively anaerobic	Aerobic	Aerobic
Growth-supporting NaCl concentrations, %	1–10	1–12-15	0.6–15	1–6	1.5–5	2–4
Maximum growth temperature, °C	35–40	35–37	35	10–26	20	37
Minimum growth temperature, °C	10	4–10	4	2	–2	13

Table 16. *Continued*

Feature	<i>Alteromonas</i>	<i>Pseudoalteromonas</i>	<i>Idiomarina</i>	<i>Colwellia</i>	<i>Glaciecola</i>	<i>Thalassomonas</i>
Hydrolysis of						
Tween-80	+	+	+	V	V	S
Chitin	-	V	V	V	-	ND
Agar	-	V	-	-	-	-
Gelatin	+	+	+	V	-	+
Utilization of						
D-Glucose, D-fructose	+	V	-	V	-	W
D-Mannose	-	V	-	ND	-	-
Sucrose	+	V	-	-	-	+
Cellobiose	+	V	-	-	-	ND
Lactose	+	V	-	-	-	-
D-Gluconate	+	V	-	V	-	-
Fumarate	-	V	-	+	V	W
Glycerol	+	-	-	V	V	-
Susceptibility to O/129 (150µg)	-	-	-	V	ND	ND
Major fatty acids	16:1ω7c; 16:0; 18:1ω7c	16:1ω7c 16:0	Iso15:0 Iso17:0	16:1ω7c 16:0 22:6 ω3	16:1ω7c 18:1ω7c 16:0	15:0 16:0 17:1ω8c
Major isoprenoid quinones	Q8	Q8	ND	Q8	ND	ND
G+C content of DNA, mol%	44-47	37-50	48-50	35-46	40-46	48.4

Symbols and abbreviations: +, positive; -, negative; S, slow reaction; W, weak reaction; V, variable reaction between strains or species; and ND, no data available.

Data from Baumann et al. (1984a, b), Bowman et al. (1998b, c), Deming et al. (1988), Ivanova et al. (2000b, 2001b), Ivanova and Mikhailov (2001a), and Macian et al. (2001).

Table 17. Phenotypic characteristics of the genera of Alteromonadaceae and related genera.

Type strain	PL	PE	PG	BPA	LPE	PA	DPG	Acyl-PG	PL-1	PL-2
<i>P. antarctica</i>	60	70.8 ± 0.9	20.2 ± 0.3	4.6 ± 0.7	1.5 ± 0.3	2.95 ± 0.4	U	U	U	U
<i>P. atlantica</i>	70	59.2 ± 2.0	31.8 ± 2.1	6.9 ± 0.1	1.1 ± 0.06	1.0 ± 0.01	U	U	2.7 ± 0.4	U
<i>P. aurantia</i>	55	63.5	14.3	2.2	16.3	3.7	U	U	U	U
<i>P. citrea</i>	75	77.5 ± 0.9	17.5 ± 0.3	1.7 ± 0.7	2.0 ± 0.2	1.35 ± 0.05	U	U	U	U
<i>P. carrageenovora</i>	70	58.5	19.6	11.4	3.0	3.2	U	U	U	4.3
<i>P. elyakovii</i>	77	60.9 ± 0.9	28.5 ± 1.0	5.6 ± 0.2	2.3 ± 0.09	2.5 ± 0.2	U	U	U	U
<i>P. haloplanktis</i>	75	71.2 ± 0.1	23.3 ± 0.4	2.5 ± 0.5	2.8 ± 0.5	0.35 ± 0.03	U	U	U	U
<i>P. luteoviolacea</i>	49	67.8	19.8	4.2	5.1	3.1	U	U	U	U
<i>P. nigrifaciens</i>	85	62.5 ± 2.4	29.7 ± 2.2	2.1 ± 0.2	1.1 ± 0.2	2.0 ± 0.06	1.4 ± 0.2	0.5 ± 0.1	U	U
<i>P. piscicida</i>	70	77.0	16.1	2.9	1.1	1.1	1.1	U	U	0.7
<i>P. rubra</i>	65	64.0	26.1	2.0	5.0	2.9	U	U	U	U
<i>P. tetraodonis</i>	80	73.4 ± 0.2	22.9 ± 0.4	2.4 ± 0.1	0.8 ± 0.2	0.45 ± 0.05	U	U	U	U
<i>P. undina</i>	60	64.4	28.4	3.1	1.4	2.7	U	U	U	U
<i>A. macleodii</i>	70	60.6 ± 1.3	37.3 ± 2.7	2.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	U	U	U	U
<i>M. communis</i>	75	66.8	21.7	1.5	1.0	3.8	2.3	1.0	U	1.9
<i>M. vaga</i>	60	59.3	11.2	7.9	2.5	5.4	4.2	3.8	U	5.7

Abbreviations: PL, phospholipids; PG, phosphatidylglycerol; BPA, 2-bromo-palmitic acid; LPE, lyso-phosphatidylethanolamine; PA, phosphatidic acid; DPG, diphosphatidylglycerol; Acyl-PG, acyl-phosphatidylglycerol; PL-1 and PL-2, unidentified phospholipids; U, undetectable.

\*Expressed as percent.

## Media

### Media

Several artificial (or synthetic) seawater (ASW) formulas have been used, e. g., ASW Formula of Lyman and Fleming (1940), modified by ZoBell (1946)

NaCl	24.32 g
MgCl <sub>2</sub> · 6H <sub>2</sub> O	10.99 g
Na <sub>2</sub> SO <sub>4</sub>	4.06 g
CaCl <sub>2</sub> · 6H <sub>2</sub> O	2.25 g
KCl	0.69 g
NaHCO <sub>3</sub>	0.20 g
KBr	0.10 g
SrCl <sub>2</sub> · 6H <sub>2</sub> O	0.42 g
H <sub>3</sub> BO <sub>3</sub>	0.027 g
Na <sub>2</sub> SiO <sub>3</sub> · 9H <sub>2</sub> O	0.005 g
NaF	0.003 g
NH <sub>4</sub> NO <sub>3</sub>	0.002 g
FePO <sub>4</sub> · 4H <sub>2</sub> O	0.001 g
Distilled water	1 liter
Adjust pH to 8.0	

### ASW Formula of MacLeod (1968)

NaCl	23.4 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	24.6 g
KCl	1.5 g
CaCl <sub>2</sub>	2.9 g
Distilled water	1 liter

While some strains grow better on ASW, containing trace compounds, others require natural seawater, especially water from the isolation source.

### Basal Medium

(BM; Baumann and Baumann, 1981)

Tris-HCl (1 M, pH 7.5)	50 or 100 ml
NH <sub>4</sub> Cl	1 g
K <sub>2</sub> HPO <sub>4</sub> · 7H <sub>2</sub> O	0.075 g
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.028 g
Distilled water	1 liter
ASW (half strength)	1 liter

Carbon sources should be added at concentration of 0.2% before sterilization (1.2 atm, 121°C, 15 min). Thermostable substances should be added by filtration to the liquefied BM cooled to 40°C. BM can be toxic for some strains which are inhibited by Tris. For strains requiring additional growth factors, amino acids (1 mg/liter, sterilization by filtration) or small amounts of yeast extract (about 10 mg), tryptone or casein hydrolysate can be added to the BM or basal medium agar, BMA, which is prepared by adding 15-20 g (Baumann and Baumann, 1981; Gauthier and Breittmayer, 1992) or up to 40 g of agar-agar per 1 liter of distilled water (Gauthier and Breittmayer, 1992). Several complex media can be used (Gauthier and Breittmayer, 1992) including yeast extract broth (YEB; BM supplemented with yeast extract [5 g/liter]) and yeast extract agar (YEA; yeast extract supplemented with agar [15 g/liter]); tryptic soya broth prepared with ASW (TSBS and its equivalent, TSBS with agar [TSAS], 15 g/liter); brain heart infusion

broth with ASW (BHIS) and BHIAS (BHIS with agar, 15 g/liter); casitone broth prepared with ASW (CBS) and its solid equivalent CAS (CBS supplemented with agar, 15 g/liter).

Marine broth (MB) and marine agar (MA), formula 2216 according to ZoBell's preparation (ZoBell, 1941, 1946) have been found suitable. Medium 2216, differs very little in composition from the medium recommended by B. Fischer (Fischer, 1894), who used fish extract in seawater ("ecological" media in modern terminology) for the isolation of marine microorganisms and who noted that many bacteria found in the sea failed to grow unless the media were prepared with seawater.

### 2216 Medium

(ZoBell, 1946)

Seawater ("aged")	1000.0 ml
Bacto-peptone	5.0 g
Ferric phosphate	0.1 g
Bacto-agar	15.0 g

After sterilization at 120°C for 20 min, medium 2216 has a pH 7.5-7.6 when in equilibrium with atmospheric CO<sub>2</sub>. Aged seawater is prepared by storing fresh seawater in glass bottles in the dark for a few weeks or longer; the water should be collected from places that are reasonably free of fresh water or terrigenous pollution.

Youshimizu and Kimura (1976) medium in modification (Mikhailov et al., 1988) can be used:

### Modified Youshimizu-Kimura Medium

Peptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
K <sub>2</sub> HPO <sub>4</sub>	0.2 g
MgSO <sub>4</sub>	0.05 g
Agar	20-40 g
Trace of iron salts	
Natural seawater	750 ml
Tap or distilled water	250 ml

After sterilization the pH is 7.5. A total of 25 g of NaCl (better, not chemically pure) per 1 liter of distilled water can be used instead of natural sea- and tap water. TSB (tryptic soya broth), BHIB (brain heart infusion broth), CB (casitone broth), MB (marine broth) and MA (marine agar) are commercially available through Difco Laboratories (Detroit, MI, United States).

Certain other media can be used for the isolation of species of  $\gamma$ -Proteobacteria covered in this chapter (Cultivation; Biotechnological Potential).

## Isolation Procedures

**SAMPLING PROCEDURES** The samples (water, sediments, organs of animals, parts of plants, etc.) should be collected aseptically. Seawater (especially for quantitative counts of culturable bacteria) can be sampled at different depths in sterile glass flasks or other containers (some-

times called bathometers). Different bathometers and problems of sampling procedures have been described by several marine microbiologists (e.g., Issatschenko, 1914; ZoBell, 1946; Baumann and Baumann, 1981; Mishustina et al., 1985, and Austin, 1988). Some authors recommended the use of a Niskin bottles (General Oceanics, Miami, FL, United States) for sampling (e.g., Tsyban et al., 1990). Sediments should be collected by core sampling using shovels and corers or by divers when possible. Bacteria adsorbed on inert surfaces and surfaces of plant, animals or some others organisms (or their teguments) have been collected using sterile cotton-tipped applicator sticks. In many cases, desorption of bacteria adsorbed to particles from sediment or seawater, disintegration of bacterial aggregates, surfaces ([bio]films), and suspension of teguments is obtained by sonication (22 kHz, 0.4 A, ~2 min) in a flask cooled in an ice bath. These samples can be used for direct isolation.

**DIRECT ISOLATION** Seawater samples are spread directly or from dilutions on solid media. Seawater (1–500 ml) can also be filtered through >0.2 micrometer pore size nitrocellulose filters, which are then placed on Petri dishes containing YEA, MA, BMA or other media with 0.1–0.2% of carbon and energy source. The volume of seawater will depend on the water source, pore size and diameter of filters, and medium used for direct isolation. After incubation of 3–7 days at 25–30°C, colonies are picked and restreaked on the same media. Species described in this chapter will generally grow at room temperature 18–25°C (10–20°C for isolation of *Colwellia* species). *Alteromonas macleodii* can be frequently obtained by direct isolation on Petri dishes containing BMA with 0.2% of lactose (Baumann and Baumann, 1981). Sediment samples are spread directly or from dilutions (approximately 1 g of sediment in 10 ml of sterile ASW or natural seawater) on the same solid media, observed for bacterial colonies and purified as described above. Similar manipulations are used for bacteria isolation by applicators and by some other methods. Agar-decomposing strains can be isolated easily on BMA without addition of organic compounds. Some *Alteromonas* strains can be isolated by using low-nutrient media, the organic-nutrient concentration of which is comparable to that found in oligotrophic marine environments, for example LON medium (0.2 mg of carbon per liter (Maeda et al., 1999; Maeda et al., 2000)).

**ENRICHMENT CULTURES** *Pseudoalteromonas denitrificans* strains have been isolated from seawater samples preincubated aerobically at 10°C in the dark in nutrient medium containing

L-glutamine, L-arginine, ethylenediamine tetraacetate (EDTA), K<sub>2</sub>HPO<sub>4</sub>, and FeSO<sub>4</sub>. Subsamples of enrichment cultures were spread on medium with the following composition: 0.5 g of Bacto-peptone, 0.5 g of Bacto-tryptone, 0.5 g of yeast extract, 200 ml of tap water, and 800 ml of seawater.

The Petri plates are incubated at 10°C, and *P. denitrificans* strains isolated from the plates and identified by their specific coloration—red, purple or blue (the color of prodigiosin and its derivatives depend on pH, medium content and temperature; Enger et al., 1987). The capacity to enzymatically degrade “marine” polysaccharides can be used for enrichment cultures of the studied bacteria. For example, carrageenanolytic strains are isolated by enrichment cultures containing carrageenan or red macroalgae, containing this polysaccharide (Lukyanov et al., 1994). During incubations of heterotrophic marine picoplankton in bottles, some bacterial groups are conspicuously favored. Bacteria of the genus *Pseudoalteromonas* rapidly multiply in substrate-amended North Sea water, whereas the densities of *Oceanospirillum* change little only. Different growth responses to substrate gradients could thus affect the competition between marine bacteria and may help to explain community shifts observed during enrichments (Eilers et al., 2000; Pernthaler et al., 2001).

No methods have been described for rapid isolation of organisms covered in this chapter. Colonies of pigmented *Pseudoalteromonas* and *Colwellia* species can be selected from isolating plates on the basis of their specific color, e.g., red or reddish, purple, purple-black, blue, violet, yellow, lemon-yellow, orange, brown and brown-black, and dark-green.

### Cultivation

*Alteromonas*, *Pseudoalteromonas*, *Idiomarina* and *Colwellia* species generally grow well on complex media in any type of container. Seawater or NaCl (sodium cations) are necessary for growth. The strains are psychrotrophic (but temperature-tolerant) or psychrophilic. Some cultures should be kept in the dark (Gauthier and Breittmayer, 1992). Good aeration is recommended, except for *P. tunicata*, *P. ulvae* and *Colwellia* species, which are facultatively anaerobic. Diverse media have been used for producers of enzymes and secondary biologically active metabolites (Mikhailov and Ivanova, 1994; Elyakov et al., 1996; Mikhailov et al., 1999).

### Preservation

Long-term preservation of species of *Alteromonas*, *Pseudoalteromonas*, *Idiomarina* and *Colwellia* can be achieved by lyophilization or by

immersion in liquid nitrogen, or cryoconservation by using low-temperature refrigerators. Lyophilized cultures can be prepared according to Baumann and Baumann (1981). Fresh colonies grown on solid media (MA, YEA, BHIS, or BMA) are suspended in 0.5 or 1 ml of a solution (ASW, 1 liter; yeast extract, 5 g; peptone, 5 g; adjusted to pH 7.5 with NaOH) and transferred aseptically into glass ampoules, which are then rapidly submerged in liquid nitrogen (or acetone-dry ice mixture), placed under vacuum for 10–12 h, then sealed and stored at 4–5°C. Freeze-dried cultures can be reconstituted by suspending the dry powder in about 0.5 ml of YEB and streaking a portion of the liquid onto YEA, while the remainder is inoculated into a tube containing 4 ml of the same medium. Growth is observed after some days of incubation at suitable temperature. After lyophilization, the cells of most species remain viable after 5–7 years of storage at 4°C (Gauthier and Breittmayer, 1992). Lyophilization does not support cell viability of *P. aurantia*, *P. citrea*, *P. luteoviolacea* and *P. rubra*. For these species, immersion in liquid nitrogen must be used for long-term preservation. A fresh culture in MB can be used (24 h of incubation at 20–25°C) or a suspension of cells grown under these same conditions on MA in one-quarter-strength sterile ASW. These suspensions are supplemented with sterile glycerol (10%, v/v) and distributed into freezing tubes (0.5 ml per 2-ml tube), which are rapidly immersed in liquid nitrogen; under these conditions, viable cells of the species were recovered after several years of storage (Gauthier and Breittmayer, 1992). These four pigmented species produce autotoxic antibiotics and they rapidly lose their viability on all media. To conserve bench cultures, it is necessary to perform weekly transfers, sometimes even 3 days for strains with a high autotoxic activity (Gauthier and Breittmayer, 1992). Most species considered in this chapter can be maintained on MA slants at 18°C (or lower temperatures) and transferred monthly. Some isolates of *A. macleodii* and *P. haloplanktis* tend to acquire growth factor requirements (amino acids) after prolonged cultivation on MA (Baumann and Baumann, 1981). The storage of many strains in ASW or MB supplemented with 30% glycerol (v/v) at –80°C can be used.

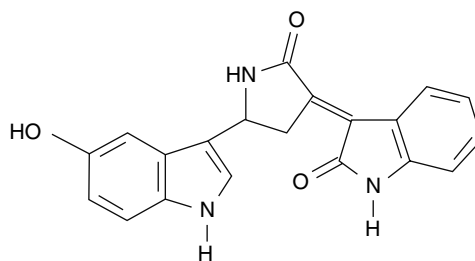
### Identification

The bacteria covered in this chapter have similar morphology, physiology, biochemistry, which facilitates their identification at the family level. Most methods used for phenotypical analysis and identification of marine bacteria have been described by Baumann and Baumann (1981) and by Gauthier and Breittmayer (1992).

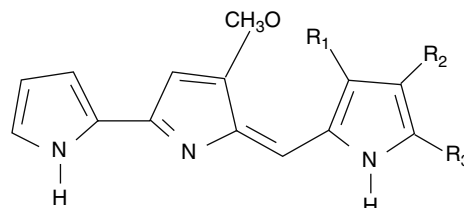
Strains are regular, straight or curved rods, which neither form spores nor microcysts. Most strains are motile by means of a polar unsheathed flagellum. Some strains of *P. luteoviolacea* and *P. denitrificans* possess a sheathed polar flagellum (Novic and Tyler, 1985; Enger et al., 1987). *Pseudoalteromonas denitrificans* sometimes forms a tuft of two to three polar flagella. Lateral flagella were described for *P. distincta* (Romanenko et al., 1995a). *Pseudoalteromonas piscicida* possess peritrichous flagella (Bein, 1954). *Idiomarina zobellii* cells are fimbriated, while *I. abyssalis* cells are enclosed in a sheath-like structure (Ivanova et al., 2000b).

On solid complex media, colonies of most *Alteromonas*, *Pseudoalteromonas*, *Idiomarina*, *Colwellia* and *Glaciecola* species cannot be differentiated in that they are small, convex with more or less regular edges, translucent whitish and yellowish (*Alteromonas* and *Idiomarina*), colorless or colored (*Pseudoalteromonas*, *Colwellia* and *Glaciecola*). Some *P. luteoviolacea*, *P. citrea* and *P. nigrifaciens* strains produce a brown-black pigment (melanoids) on complex solid media. Colonies of pigmented *Pseudoalteromonas*, *Glaciecola* and *Colwellia* strains can be differentiated by color. *Pseudoalteromonas citrea* and *P. aurantia* produce yellow and orange noncarotenoid pigments. *Pseudoalteromonas luteoviolacea* produces intracellular violacein, and *P. denitrificans*, *P. rubra* and *P. bacteriolytica*, prodigiosin:

Violacein



Prodigiosin

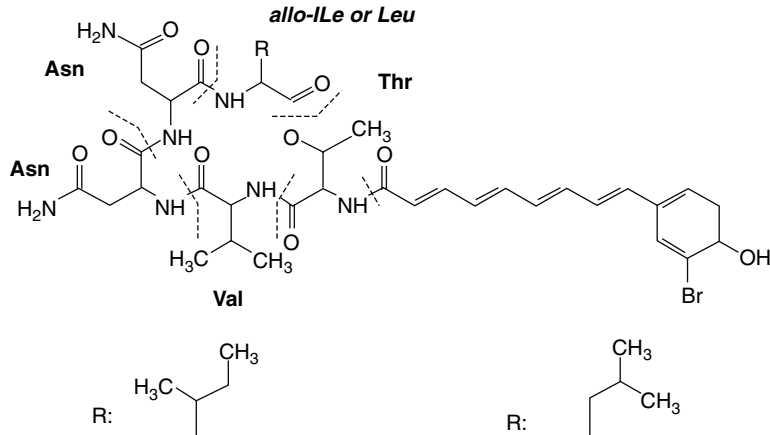


(R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> = H or alkyl)

The strain *Pseudoalteromonas maricaloris* KMM 636<sup>T</sup> (Ivanova et al., 2002b), isolated

from marine sponge *Fascaplysinopsis reticulata*, produce yellow-orange chromopeptides, bromoalterochromides (T. A. Kuznetsova and H. Laatsch, personal communication):

#### Bromoalterochromides A and B



The main differentiating characteristics of species assigned to *Alteromonas* and related genera are shown in Tables 3, 5, 6, 9, 11, 13, 15, 16 and 17. Identification of isolates can be done by comparing their characteristics with those of type strains, compiled in Table 4.

All species need seawater for growth and have strict Na<sup>+</sup> requirement. Growth-supporting NaCl concentrations are 1–10% for *Alteromonas*, 1–12–15% for *Pseudoalteromonas*, 0.6–15% for *Idiomarina*, 1–6% for *Colwellia* and 1.5–5% for *Glaciecola*. The presence of Na<sup>+</sup> and K<sup>+</sup> are essential for the oxidation of exogenous substrates, including intermediates of the tricarboxylic acid, while the activities of the enzymes of this cycle are independent of these ions (MacLeod et al., 1958; MacLeod and Hori, 1960). Also, Na<sup>+</sup> ions play a role in the functioning of several permeation systems, Na-dependent transport systems of amino acids, maintenance of the integrity of the cell wall, and some other functions investigated by MacLeod and coworkers (Gauthier and Breittmayer, 1992).

Except for *P. tunicata* and *P. ulvae*, all species of the *Alteromonas*, *Pseudoalteromonas*, *Idiomarina* and *Glaciecola* genera are aerobic. *Colwellia* species, except *C. maris*, are facultatively anaerobic. All species of the genera *Alteromonas*, *Pseudoalteromonas* and *Idiomarina* are resistant to O/129 (except for *P. tunicata* and *P. ulvae*), and to lincomycin, but are sensitive to erythromycin.

Except for *P. denitrificans* and some strains of *P. haloplanktis*, none of the species produces nitrites from nitrates. None of species has a constitutive arginine dihydrolase system or accumulates polyhydroxybutyrate as intracellular storage product.

*Alteromonas* strains can grow between 10 and 35–40°C, *Pseudoalteromonas* strains from 4 to

35–40°C, *Idiomarina* strains from 4 to 35°C, *Colwellia* strains from 2 to 25°C, and *Glaciecola* species from –2 to 20–25°C.

The ability to oxidize or ferment D-glucose is an important diagnostic test. The phenol-red-containing Leifson's O/F medium adapted to marine bacteria gives reliable results (Leifson, 1963).

*Alteromonas*, *Pseudoalteromonas*, *Idiomarina*, *Colwellia* and *Glaciecola* species can use several organic compounds as sole source of carbon and energy, including carbohydrates, especially D-glucose and D-fructose (except for strains of *Idiomarina*, all *Glaciecola* strains and some *Pseudoalteromonas* species), alcohols, carboxylic acids, amino acids, and aromatic compounds. *Alteromonas macleodii*, *Pseudoalteromonas haloplanktis*, and *P. espejiana* use D-glucose and D-fructose through an inducible Entner-Doudoroff pathway. The API system (Analytab Products, Plainview, NY) and other commercial systems have been used to analyze the consumption of organic compounds as the sole or main carbon and energy source, and determine some other biochemical properties of bacteria.

The cellular fatty acid profile of the bacteria is a valuable taxonomic marker. Major fatty acids are: for *Alteromonas macleodii* and *Pseudoalteromonas*, 16:0 and 16:1 $\omega$ 7c; for *Idiomarina*, *i*15:0 and *i*17:0; for *Glaciecola*, 16:0, 16:1 $\omega$ 7c and 18:1 $\omega$ 7c; and for *Colwellia*, 22:6 $\omega$ 3 (Table 106).

## Ecology of *Alteromonas* and Related Bacteria

The bacteria of this chapter are widely distributed obligate marine bacteria. Habitats and ecological niches of these microorganisms are



diverse (Austin, 1988; Fenical, 1993; Mikhailov and Ivanova, 1994; Mikhailov et al., 1999; Ivanova and Mikhailov, 2001a; Ivanova et al., 2001b). Sources of isolation and the geographic distribution of species of *Alteromonas*, *Glaciicola*, *Pseudoalteromonas*, *Colwellia*, *Idiomarina* and *Thalassomonas* are shown in Table 4. This group are common inhabitants of the coastal, open and deep waters of the seas and oceans (Pacific, Atlantic, Indian, Arctic and Antarctic). Members of *Alteromonas*-like bacteria are isolated from a variety of biotopes, i.e., the surface of biological and non-biological objects, sediments, seawater, intestines of marine animals, sea ice, etc. Bacteria of this group, with the exception of type strain *P. nigri-faciens*, have never been isolated from soil or other terrestrial biotopes because of their obligate requirement for Na<sup>+</sup> (≥100 mM NaCl). Bacteria of these genera are heterotrophs, chemorganotrophs, psychrotrophs, slightly or moderately halophilic.

Because tentative identification of pigmented antibiotic-producing species of pseudoalteromonads is relatively simple, their distribution in the coastal waters of the Mediterranean Sea has been intensively studied. According to Gauthier et al. (1975) and Lemos et al. (1985), strains of *P. rubra*, *P. luteoviolacea*, *P. citrea* and *P. aurantia* are constantly isolated from seawater samples in autumn during phytoplankton blooms and represent more than 5% of the total culturable microorganisms. Pigmented *Pseudoalteromonas*-like bacteria that produced antibiotics and autotoxins were isolated from Australian (Gavrilovic et al., 1982) and Japanese (Ezura et al., 1988) coastal waters, from sediment samples, phyto- and zooplankton, and marine invertebrates of the Sea of Japan (Nair and Simidu, 1987; Ivanova et al., 1998b). Interestingly, prodigiosin-producing strains of *P. denitrificans* were isolated only from the samples of seawater collected 90–100 m from Norway's fiords and have never been found in other coastal waters and sediments (Enger et al., 1987).

Bacteria of the genus *Pseudoalteromonas* are well-known producers of numerous biologically active substances. Some of these activities have been exploited commercially (Biotechnological Potential). Synthesis of some secondary metabolites is controlled by bacterial attachment to certain substrates (surfaces) with defined physical and chemical properties (Ivanova et al., 1998c). It is ecologically important that these bacteria can also regulate the morphogenesis of marine invertebrates (Letz and Wagner, 1993) by inducing the larval settlement of the starfish *Acan-taster planci*, which is a predator of the Great Barrier Reef corals and larvae of some other animals (Johnson et al., 1991). It has also been

reported that these bacteria are pathogenic for invertebrate larvae (Colwell and Sparks, 1967) and algae *Laminaria japonica* (Sawabe et al., 1998b). Bdellovibrios are intracellular parasites of *Vibrio* spp. but not of *P. atlantica* (Sutton and Besabt, 1994).

## Biotechnological Potential

### *Alteromonas* and Related Bacteria as Producers of Enzymes

Recent investigations have demonstrated marine *Alteromonas*-like organisms are a rich source of enzymes of industrial potential such as proteinases, endoglucanases, glycosidases, chitinases, agarases, nucleases and tyrosinases. Producers of highly active and specific hydrolases including alkaline phosphatase (Fedosov et al., 1991), β-1,3-glucanase (laminaranase; Sova et al., 1994), polyuridyl-endoribonuclease (Romanenko et al., 1995b), RNases (Ivanova et al., 1994b; Plisova et al., 1996), α-*N*-acetylgalactosaminidase (Bakunina et al., 1994), α-galactosidase (Bakunina et al., 1996), β-*N*-acetylglucosaminidases (Ivanova et al., 1998a) have been described. *Pseudoalteromonas* strains were found to synthesize restrictases (Dedkov et al., 1990), nucleoside-kinases (Terent'ev et al., 1998), amylase (Gavrilovic et al., 1982; Aghajari et al., 1998), phospholipase and lipophospholipase (Cadman and Eichberg, 1983) and other enzymes (Ivanova and Mikhailov, 1992a; Mikhailov and Ivanova, 1994).

Many bacteria from the sea (ZoBell and Rittenberg, 1938; Gooday, 1990; Gooday, 1994), including *Alteromonas-Pseudoalteromonas*, are able to produce chitinolytic enzymes, chitinases and β-*N*-acetylglucosaminidases (chitobiasis). The search for bacterial producers of chitinases resulted in the isolation from the bottom deposit sample (depth 5 m) of the Sagami Bay, Japan, of *Alteromonas* strain O-7, which possesses a highly active extracellular chitinase Chi-A (Tsujiibo et al., 1991). The amino acid sequences of this chitinase was homologous to described chitinases from terrestrial bacteria such as *Serratia marcescens* and *Bacillus circulans*. However, chitinase ChiA showed higher activity and stability in seawater owing to the presence of sodium, potassium, calcium and magnesium ions (Tsujiibo et al., 1992). Subsequent investigations revealed that chitinolytic system of the strain O-7 included three chitinases (ChiA, ChiB, and ChiC) and three *N*-acetylglucosaminidases (Tsujiibo et al., 1993; Tsujiibo et al., 1995; Tsujiibo et al., 1998). The cluster of three closely linked chitinase genes organized in the order *chiA*, *chiB* and *chiC*, with the same transcriptional direction, and two unlinked genes, *chiP* and *chiQ*, involved

in chitin degradation in *Pseudoalteromonas* sp. S91, were cloned, sequenced and characterized. The deduced amino acid sequences revealed that ChiA, ChiB and ChiC exhibited similarities to chitinases belonging to family 18 of the glycosyl hydrolases, while ChiP and ChiQ belonged to family 20 (Techkarnjanaruk et al., 1997; Techkarnjanaruk and Goodman, 1999). Reports reveal that only a restricted number of alteromonad and pseudoalteromonad strains possess chitinases (Ivanova and Mikhailov, 1992a; Ivanova et al., 1992b).

Polysaccharide agar-agar (also known as agar, which is a mixture of agarose and agaropectin) is available in red algae cell walls. Agar can be degraded by both marine and other bacteria using two pathways which differ in the specificity of the cleaving enzymes. The first pathway is based on an extracellular  $\beta$ -agarases (Morrice et al., 1983a; Morrice et al., 1983b). Endo  $\beta$ -agarase I cleaves the  $\beta$ -(1,4) linkages of large agar polymers to a mixture of oligosaccharides with neoagarotetraose as a main and final product. Oligosaccharides are then hydrolyzed by the cell-bound exo  $\beta$ -agarase II, yielding neoagarobiose. Neoagarobiose is hydrolyzed intracellularly to 3,6-anhydro-L-galactose and galactose by neoagarobiose hydrolase (Day and Yaphe, 1975). The second pathway involves the cleavage of  $\alpha$ -(1,3) linkages of agarose by extracellular  $\alpha$ -agarases (Potin et al., 1991; Potin et al., 1993), yielding oligosaccharides from the agarobiose series, which contain D-galactose at the non-reducing end (Vera et al., 1998). *Pseudoalteromonas atlantica* strains are an industrial source of agarases (Yaphe, 1957; Akagawa-Matsushita et al., 1992b). An extracellular agarase was isolated from *Alteromonas* sp. C-1 (Leon et al., 1992) characterized by the molecular mass (m.m.) of 52 kDa, optimum pH 6.5, sensitivity to NaCl, and hydrolysis of agar with neoagarotetraose as a main product. Among pseudoalteromonads, producers of  $\beta$ -agarases have been characterized (Romanenko et al., 1994a; Romanenko et al., 1994b, 1994b). The enzyme from *Pseudoalteromonas antarctica* N-1 hydrolyzed  $\beta$ -1,4-glycosidic linkages of agar, yielding neoagarotetraose and neoagarohexaose as main products, and exhibited maximal activity at pH 7 (Vera et al., 1998).

$\alpha$ -D-Galactosidase cleaves the  $\alpha$ -D-galactopyranosyl residue from the nonreducing end of various oligosaccharides, branched polysaccharides (galactans and galactomannans), as well as corresponding glycosides and various natural glycoconjugates. Marine bacteria, including *Alteromonas*-like strains, have been screened for  $\alpha$ -galactosidases,  $\alpha$ -N-acetylgalactosaminidases, and  $\beta$ -N-acetylglucosaminidases (Bakunina et al., 1994; Bakunina et al., 1996; Ivanova et al.,

1998a).  $\alpha$ -Galactosidase and agarase were isolated and partially purified from the agarolytic strain of *Pseudoalteromonas citrea* KMM 188 associated with a mussel. Enzymes were characterized by a pH optimum of 6.0–7.5 and temperature stability of up to 30°C (Bakunina et al., 1996). *Pseudoalteromonas* sp. KMM 701 was found to produce  $\alpha$ -galactosidase capable of inactivating the group specificity of B erythrocytes (group III) of human blood and of A erythrocytes (group II). The enzyme was stable at 20°C for 24 h at high NaCl concentrations, and had a pH optimum between 6.7 and 7.7. The enzyme was 4-fold more efficient than the  $\alpha$ -galactosidase from green coffee beans. The molecular mass of the enzyme was  $195 \pm 5$  kDa. The  $\alpha$ -galactosidase was denatured by urea and guanidine hydrochloride. A sulfhydryl was essential for its catalytic activity, which did not depend on the presence of metal ions (Bakunina et al., 1998).

The cold-adapted  $\beta$ -galactosidase from *Pseudoalteromonas haloplanktis* TAE 79 was purified to homogeneity. The enzyme shared structural properties with *Escherichia coli*  $\beta$ -galactosidase (comparable subunit mass, 51% amino sequence identity, conservation of amino acid residues involved in catalysis, similar optimal pH value, and requirement for divalent metal ions) but was characterized by a higher catalytic efficiency on synthetic and natural substrates, a shift of apparent optimum activity toward low temperatures, and lower thermal stability. Assays of lactose hydrolysis in milk demonstrated that *P. haloplanktis*  $\beta$ -galactosidase can outperform the current commercial  $\beta$ -galactosidase from *Kluyveromyces marxianus* var. *lactis*, suggesting that the cold-adapted  $\beta$ -galactosidase could be used to hydrolyze lactose in dairy products processed in refrigerated equipment (Hoyoux et al., 2001). Psychrophilic enzymes produced by cold-adapted microorganisms display a high catalytic efficiency associated with high thermosensitivity. The application of cold-adapted enzymes offers considerable potential to the biotechnology industry (e.g., in the detergent and food industries), for the production of fine chemicals and bioremediation processes (Gerday et al., 2000).

*Pseudoalteromonas carrageenovora* is producer of carrageenases which decompose carrageenans, complex polysaccharides contained in the cell walls of *Rhodophyta algae* (Yaphe and Baxter, 1955; Yaphe, 1957; Yaphe, 1962). *Pseudoalteromonas carrageenovora* and 10% of *P. atlantica* strains are able to hydrolyze carrageenans as well as alginates (Akagawa-Matsushita et al., 1992b).

*Pseudoalteromonas* strains from the brown algae *Fucus evanescens*, *Chorda filum*, and the

sea cucumber *Sticopus (Apostichopus) japonicus* produce fucoidanases (fucoidan-hydrolases), which hydrolyze fucoidan, a polysaccharide of brown algae (Bakunina et al., 2000).

A laminaran-induced  $\beta$ -1,3-glucanase (laminaranase) with m.m. 40 kDa, temperature optimum 45°C, and pH optimum 5.5 was isolated from the type strain KMM 162 of *Pseudoalteromonas elyakovii*. Hydrolysis products of laminaran were laminaran oligosaccharides and glucose. The enzyme had transglycosylating activity and was endo-specific. Chemical modification indicated that tryptophan, histidine, and dicarboxylic acid residues were important for enzymatic activity (Sova et al., 1994).

Some bacterial strains are capable of expressing alginate lyase. Alginate is a linear polymer consisting of  $\beta$ -D-mannuronic and  $\alpha$ -L-guluronic acid residues and is a part of the cell walls of brown algae. Strain H-4 of *Alteromonas* sp. produced extra- and intracellular alginolytic enzymes and utilized alginate as the sole source of carbon. Extracellular alginate lyase of strain H-4 was found to be a novel enzyme with a wide substrate specificity, m.m. of 32 kDa and an optimum pH of 7.5. The enzyme was unstable at temperatures above 30°C and in acidic media. Its activity increased in the presence of magnesium, sodium, and potassium ions (Sawabe et al., 1997; Sawabe et al., 1998b). Detailed taxonomic investigation of these alginolytic bacteria isolated from spot-wounded fronds of *Laminaria japonica* allowed them to be assigned to *Pseudoalteromonas elyakovii* (formerly *Alteromonas elyakovii*; Ivanova et al., 1996b; Sawabe et al., 2000).

Nucleic acids are widespread compounds in the sea environment as described by the presence of a large number of bacteria producing DNAses and RNAses, including proteobacteria (Wei et al., 1983; Przykorska et al., 1988; Austin, 1988; Romanenko and Mikhailov, 1992; Ivanova et al., 1994b; Plisova et al., 1996). Strain KMM 223, producer of polyuridyldendoribonuclease, exhibits a unique polyuridylic acid-hydrolyzing activity. This enzyme specifically degraded poly(U) by an endonuclease mechanism that results in oligonucleotides with terminal 5'-phosphate (Romanenko et al., 1995b; Plisova et al., 1996).

Some *Pseudoalteromonas haloplanktis* strains produce isoschizomeric endonucleases of the known restrictases *Sau961* and *KpnI* (Dedkov et al., 1990). Isoschizomers of endonucleases *XbaI*, *EcoRII*, *BalI*, *NaeI* have also been found in *Pseudoalteromonas* strains (Romanenko et al., 2001).

An alkaline phosphatase (AP) has been characterized from strain KMM 162 of *Pseudoalteromonas elyakovii* that was isolated from the

coelomic liquid of the mussel *Crenomytilus grayanus*. The AP from the strain KMM 162 had an activity of 7000 units/mg of protein at pH 7.5–11.0. The activity of the enzyme depended on concentration of ions in the test assay. Replacement of glucose with laminaran ( $\beta$ -1,3-glucan) in culture medium resulted in the synthesis of both AP and laminaranase with high activities (Fedosov et al., 1991; Ivanova et al., 1991). Other AP-producing *Alteromonas*-like proteobacteria have been described (Ivanova et al., 1992c; Terent'ev et al., 1994).

*Alteromonas* and *Pseudoalteromonas* strains synthesize highly active proteolytic enzymes (Austin, 1988; Ivanova and Mikhailov, 1992a; Ivanova et al., 1992d). A serine-protease (Dohmoto and Miyachi, 1991) has been reported to occur in a strain isolated from the mussel *Mytilus edulis*. The proteinase showed high degrading activity of mussel thread (byssus). *Pseudoalteromonas peptidolytica* (Venkateswaran and Dohmoto, 2000) has been described as a new proteinase-producing bacterium.

A strain of *P. piscicida*, isolated from an algae of the Black Sea, displayed thrombolytic activity. This strain excreted an exoprotease, the lyophilized enzyme (1 mg/ml) of which lysed thrombs (clots) of human blood in vitro after 5 min of incubation. After the incubation, the activity was completely depressed (Demina et al., 1990). The enzyme showed direct fibrinolytic activity since plasminogen was not activated.

*Alteromonas haloplanktis* strain S5B produced an aminopeptidase and an extracellular trypsin-like protease. The latter represented an alkaline protease, the activity of which increased with calcium ions. The specific properties of the isolated enzyme were thermal lability and activity at low temperatures. These features play an important role in the destruction of fish proteins in the sea environment (Odagami et al., 1994).

Bacteria covered in this chapter also produce oxidoreductases. Tyrosinases can exhibit two enzymatic activities, namely cresolase and catecholase, which catalyze the conversion of tyrosine via L-3,4-dihydroxyphenylalanine (L-DOPA) to melanin. L-DOPA is known an effective agent in the treatment of Parkinson's and some other diseases. Model experiments have revealed the involvement of tyrosinases from marine bacteria in the metamorphosis and adhesions of oyster larvae of *Crassostrea virginica* and other invertebrates (Bonar et al., 1986). Screening of *Pseudoalteromonas* strains, both associated with invertebrates and free living, has shown a great number of strains (up to 40%) that express high level of tyrosinase activity. Melanogenic pseudoalteromonads represented by *P. citrea* and *P. distincta* were characterized by a high intracellular general and

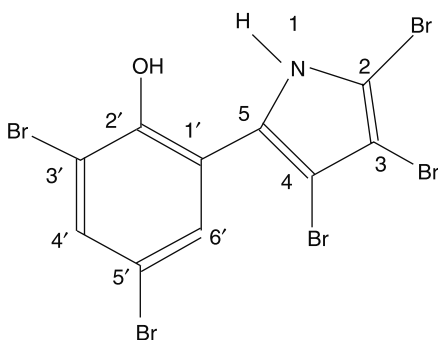
specific tyrosinase activities.  $\beta$ -Tyrosinase with the m.m. of 26.3 kDa was isolated from cells of *P. distincta* KMM 638<sup>T</sup> (Romanenko et al., 1994c).

### Secondary Metabolites of Pseudoalteromonads and Related Bacteria

Microorganisms of the marine environment are capable of synthesizing diverse bioactive secondary metabolites. De Giaxa (1889) was the first to report on marine bacteria with properties antagonistic to the causal agents of anthrax and cholera. Later, ZoBell and his coworkers (ZoBell and Upham, 1944; ZoBell and Upham, 1946; Rosenfeld and ZoBell, 1947), and other researchers (Baam et al., 1966a; Baam et al., 1966b; Baslow, 1969) based their studies on these earlier findings.

The first report on the antibiotic activity of *Alteromonas*-like bacteria revealed the ability of a certain "variant" of *Pseudomonas piscicida* to inhibit the growth of yeast (Buck and Meyers, 1966). In 1966, Burkholder et al. (1966) reported about the isolation of the brominated pyrrole antibiotic (2,3,4-tribromo-5[1'-hydroxy-2',4'-dibromophenyl]-pyrrol) from "*Pseudomonas bromoutilis*." This unique compound, consisting of more than 70% bromine (Lowell, 1966), was found later in *P. luteoviolacea* and named "pentabromopseudilin" (Laatsch and Pudleiner, 1989; Hanefeld et al., 1994). Pentabromopseudilin was active against Gram-positive test cultures in low concentrations. It was the first antibiotic from an obligately marine bacterium for which a novel chemical structure was reported. Two strains of "*P. bromoutilis*" were isolated from turtle grass *Thalassia* sp. collected in tropical waters near Puerto Rico. "*Pseudomonas bromoutilis*" had a respiratory type of metabolism, were catalase negative, and formed yellow colonies with a dark trace on solid media. Subsequent biochemical characteristics indicated that these strains belonged to either *Alteromonas* or *Pseudoalteromonas*.

Pentabromopseudilin

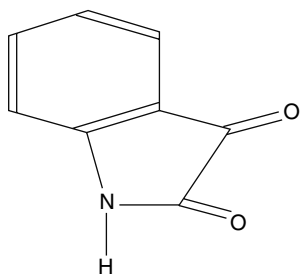


In 1974, Anderson isolated from a seawater sample (obtained from the northern area of the Pacific Ocean) a purple-colored bacterium resembling the known *Chromobacterium marinum* (Anderson et al., 1974). The phenotypic characteristics of the isolate were similar to those of the genera *Alteromonas* or *Pseudoalteromonas*. This strain synthesized several antimicrobial compounds, which were extracted from the cells by ethyl acetate: tetrabromopyrrol, hexabrom-2,2'-bipyrrrol and 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrol. Tetrabromopyrrol, the most active of the compounds, inhibited the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* (Wratten et al., 1977).

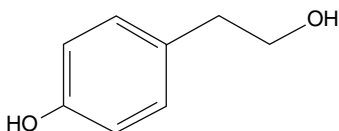
A collection of violet-pigmented strains of *Pseudoalteromonas* (*Alteromonas*) *luteoviolacea* was studied by Gauthier and Flatau (1976c). They isolated two brominated compounds together with polyanionic weakly cell-bound polysaccharides, which diffused into the medium. The substances inhibited the growth of Gram-positive microorganisms, such as *Bacillus firmus*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*, but were less active against Gram-negative bacteria, e.g., *Morganella morganii* and *Shigella dysenteriae*. Some other pigmented species of *Pseudoalteromonas* genus, *P. rubra*, *P. citrea* and *P. aurantia*, were also able to synthesize autotoxic high molecular weight antibiotic substances, active against Gram-positive and Gram-negative microorganisms (Gauthier, 1976a; Gauthier, 1976b; Gauthier, 1977; Ballester et al., 1977; Gauthier and Breittmayer, 1979). Three metabolites active against protist *Paramecium caudatum* were later isolated from *Pseudoalteromonas* (*Alteromonas*) *luteoviolacea* (Kamei et al., 1986).

Members of Fenical's group from Scripps Institution of Oceanography have noticed that shrimps (*Palaemon macrodactylus*) that usually dwell in estuaries were resistant to pathogens, including the known pathogen of the crustacean *Lagenidium callinectes*. Shrimp eggs are colonized by bacterial cells that probably protect eggs by producing antibiotic substances. The associative bacteria were isolated in axenic culture and identified as *Alteromonas* sp. During laboratory cultivation, *Alteromonas* sp. synthesized the fungicidal agent 2,3-indolinedione known also as "isatin." Removal of epibiotic alteromonads led to the death of shrimp embryos in contrast to embryos reinoculated with *Alteromonas* or treated by isatin (Gil-Turnes et al., 1989). Isatin is used for synthesis of indigoid dyes and its derivatives as fungicides, herbicides and other preparations.

## 2,3-Indolinedion (isatin)



Also, Fenical and collaborators showed that an unidentified Gram-negative bacterium, probably a pseudoalteromonad that coated eggs of the lobster *Homarus americanus*, was able to synthesize a large amount of 2-*p*-hydroxyphenylethanol (tyrosol), which inhibited the growth of *L. callinectes* (Gil-Turnes and Fenical, 1992). Earlier, tyrosol was determined to be a metabolite of terrestrial microscopic fungi.

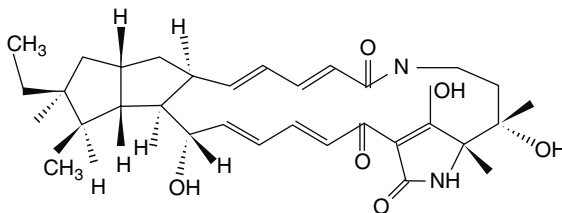
2-*p*-Hydroxyphenylethanol (tyrosol)

Bacteria of *Alteromonas* genus were found to influence some species of red microalgae (during “blooming”) or red tide plankton. *Alteromonas* sp. 9066-46 was isolated from coastal seawater of the Sea of Japan and displayed an inhibitory effect on the growth of the red algae *Gymnodium nagasakiense*. When cell suspensions of strain 9066-46 from the early and late logarithmic phases were added to the microalgae culture, growth of the algae was considerably decreased. The same specific algicidal activity was obtained with ethylacetate extracts of *Alteromonas* sp. 9066-46. Algicidal effects against other microalgae species were not found (Sugahara et al., 1992).

The bacterium *Alteromonas* sp., synthesizing tetracyclic alkaloid alteramide, was isolated from the sponge *Halichondria okadai* found in the Sea

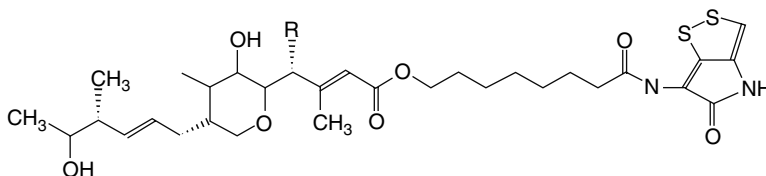
of Japan (Shigemori et al., 1992). Alteramide is a representative of the new class of macrocyclic lactams, which undergoes an intramolecular photochemical cyclization with the formation of a hexacyclic product. Alteramide showed a cytotoxic activity against leukemia P-388, lymphoma L 1210 and epidermal carcinoma KB cells in vitro at the concentration of 0.1, 1.7 and 5.0  $\mu\text{g/ml}$ , respectively. The cyclization product had no cytotoxic activity

## Alteramide

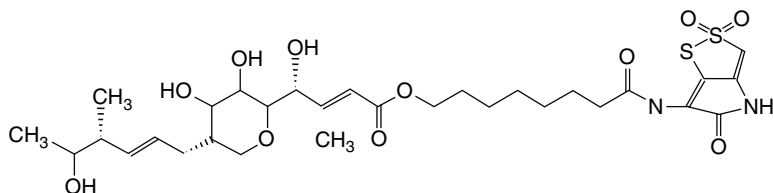


Isolated from the seawater bacterium “*Alteromonas rava*” SANK 73390 (Kodama et al., 1993), a new antibiotic thiomarinol (Shiozawa et al., 1993; Shiozawa and Takahashi, 1994) was described. Thiomarinol is a derivative of two types of antibiotics, pseudomonic acid and pyrroline antibiotics, containing a disulfide bridge. Activity of thiomarinol against Gram-positive bacteria, including antibiotic-tolerant *Staphylococcus aureus* strains and Gram-negative bacteria, was considerably higher than those of pseudomonic acid and acylpyrrolitin. Later, thiomarinol was renamed “thiomarinol A” (Shiozawa et al., 1995) when thiomarinols B and C were described. Thiomarinol B has the greatest antimicrobial activity among the thiomarinols, whereas C is less active than A. The minimum inhibitory concentration of thiomarinol B towards *S. aureus* 535 (MRSA) was  $<0.01 \mu\text{g/ml}$ . It should be noted that thiomarinol A had been isolated earlier from *Alteromonas* sp., as one of the two new derivatives of pseudomonic acid (Stierle and Stierle, 1992), but the absolute configuration of this compound was not determined. Thiomarinols D, E, F and G were described later (Shiozawa et al., 1997).

Thiomarinol A (R = OH); Thiomarinol C (R = H)

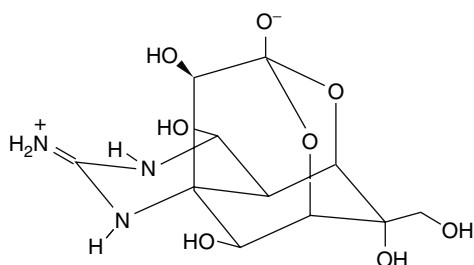


## Thiomarinol B



Several research groups reported that known metabolites (such as tetrodotoxin, saxitoxin, eicosapentaenoic acid, tetrabromodiphenyl ethers, etc.) are really synthesized by symbiotic bacteria but not by their hosts (such as marine fish, mollusks and other animals; Noguchi et al., 1986; Yasumoto et al., 1986; Yazawa et al., 1988; Kodama et al., 1989; Elyakov et al., 1991). Tetrodotoxin is one of the strongest natural neurotoxins. It has valuable pharmacological properties as an analgesic, e.g., an agent against asthma, arthritis, impotence and other diseases. The assumption that tetrodotoxin is synthesized by symbiotic microorganisms was confirmed by toxin production of *Pseudomonas*, *Vibrio* and other Gram-negative bacteria isolated from sea animals (Noguchi et al., 1986; Yasumoto et al., 1986). The new species *Alteromonas tetraodonis*, isolated from skin slime of Fugu fish, was described by Simidu and collaborators (Simidu et al., 1990). It was also demonstrated that tetrodotoxin was synthesized by free-living, both sea and fresh water Gram-negative and Gram-positive bacteria (Simidu et al., 1995). The ability to produce tetrodotoxin by bacteria, however, is currently under discussion (Matsumura, 2001; Kim and Kim, 2001).

## Tetrodotoxin

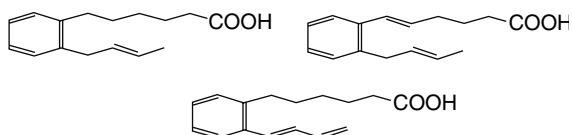


By screening 3000 bacterial strains from coastal waters of Japan, producers of enzyme inhibitors have been found. *Alteromonas* sp. B-10-31 produced low molecular weight (1.5–1.7 kDa) peptides called “marinostatins,” which act as an inhibitor of proteases (subtilisin). Three categories of marinostatins were isolated: A, B (B-1 and B-2) and C (C-1 and C-2). The main components were marinostatins B and C,

containing alanine, arginine, aspartic and glutamic acids, methionine, phenylalanine, proline, serine, threonine and tyrosine. Marinostatins were stable at pH 2–6, specific to serine proteases such as subtilisin, bull  $\alpha$ -chymotrypsin, pancreatic protease, proteinase K, and inhibited by ions of  $\text{Co}^{2+}$  and  $\text{Fe}^{2+}$ . High molecular inhibitors named “monostatins” were also isolated from strain B-10-31. Monostatins are glycoproteins (about 20 kDa), stable at pH 2–12 and specific to thiol proteases. Monostatins are inhibited by ions of  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  (Imada et al., 1985a; Imada et al., 1985b; Imada et al., 1985c; Imada et al., 1986a; Imada et al., 1986b).

Leupeptins A, B and C from *Alteromonas* sp. are thiolproteinase inhibitors (Hamato et al., 1984). These possess at the C-terminus an aldehyde of arginine, followed by amino acids acetyl-leucine and valine, acetyl-leucine and isoleucine, acetyl-leucine and leucine for leupeptins A, B and C, respectively. Similar aldehyde-containing peptides were isolated earlier only from various species of actinomycetes (actinobacteria).

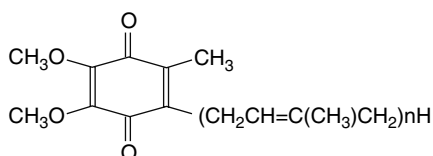
Scientists of the Roche Research Institute (Australia) isolated from seawater a strain of *Pseudoalteromonas* (*Alteromonas*) *rubra*, which in liquid culture synthesized a series of unusual  $\text{C}_{16}$  aromatic acids. These compounds had myorelaxant properties (Holland et al., 1984).

 $\text{C}_{16}$  aromatic acids of *Pseudoalteromonas rubra*

Isoprenoid quinones, and in particular, ubiquinones (coenzymes Q) are components of the respiratory chain where they participate in the transport of electrons and participate in oxidative phosphorylation. The biological activity of quinones allows them to be used as antioxidants in anemia, muscular dystrophy, and cardiovascular insufficiency. Numerous strains of terrestrial pseudomonads have been pro-

posed as sources of ubiquinones. However, also marine bacteria such as *Alteromonas* and *Marinomonas* containing ubiquinones Q<sub>8</sub>, *Deleya* (*Halomonas*) and marine *Pseudomonas* spp. containing Q<sub>9</sub>, *Shewanella* spp. containing ubiquinones and menaquinones, and *S. putrefaciens* containing methylmenaquinones (Akagawa-Matsushita et al., 1992a) could be used as sources of ubiquinones.

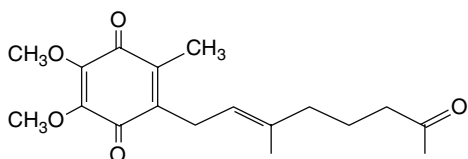
#### Ubiquinones



*Alteromonas* sp. KK 10304, associated with the marine sponge *Halichondria okadai*, effectively inhibited settling of the cyprids of the barnacle, *Balanus amphitrite*. Bioassay-guided isolation indicated ubiquinone-8 as an effective inhibitor of cyprid settling (Kon-ya et al., 1995).

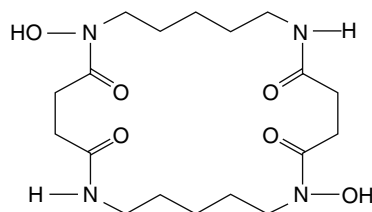
From the surface of the cyanobacterium *Microcoleus lyngbiiaceus* (from waters off the coast of Puerto Rico) four pseudoalteromonad-like bacteria (one violet, one red, and two yellow) were isolated. All of them synthesized the same quinone (20 mg/liter of medium) with a strong fungicidal activity (Fenical, 1993).

Quinone is synthesized by associated bacteria of *M. lyngbiiaceus*



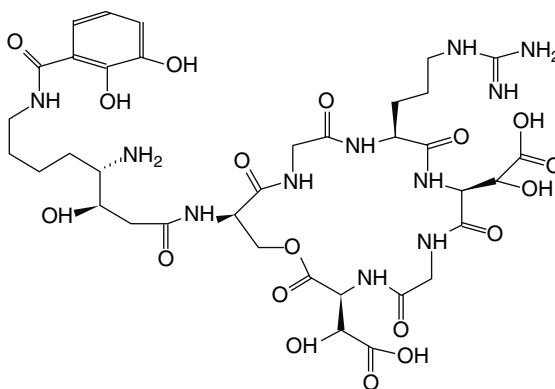
Several reports indicate the production of siderophores by marine bacteria. The first described structure was bisucaberin, isolated from *Pseudoalteromonas* (*Alteromonas*) *haloplanktis* SB-1123 (Kameyama et al., 1987; Takahashi et al., 1987). The strain was isolated from sediments (at a depth of 3300 m) obtained off the coast of the Japanese prefecture of Aomori and in less than 2 days cultivation at 27°C on medium containing fishmeal and maltose. Under these conditions, the strain synthesized 700 mg of bisucaberin per liter of medium. This siderophore has the unique biological property to make tumor cells sensitive to the cytolytic effect of macrophages. The cytotoxic effect of bisucaberin was demonstrated on tumor cells of fibrosarcoma 1023 (Kameyama et al., 1987).

#### Bisucaberin



Extraordinary affinity for iron is displayed by the alterobactins A and B isolated from *Pseudoalteromonas* (*Alteromonas*) *luteoviolacea*. These cyclic depsipeptides contain two unusual amino acids—L-treo-β-hydroxyaspartic and (3R, 4S)-4,8-diamino-3-hydroxyoctanoic acids. Alterobactin has been chemically synthesized (Deng et al., 1995).

#### Alterobactin A



### Literature Cited

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## Nonmedical: *Pseudomonas*

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### Introduction

*Pseudomonas* comprises a genus of species capable of utilizing a wide range of organic and inorganic compounds and of living under diverse environmental conditions. Consequently, they are ubiquitous in soil and water ecosystems and are important as plant, animal and human pathogens (Palleroni, 1992; Schroth et al., 1992). The genus *Pseudomonas* is well known for its metabolic versatility and genetic plasticity. The species of *Pseudomonas*, in general, grow rapidly and are particularly renowned for their ability to metabolize an extensive number of substrates, including toxic organic chemicals, such as aliphatic and aromatic hydrocarbons. Strains of *Pseudomonas* species are often resistant to antibiotics, disinfectants, detergents, heavy metals, and organic solvents. Some strains have been confirmed to produce metabolites that stimulate plant growth or inhibit plant pests.

*Pseudomonas* was already recognized as a complex collection of a large number of described species when the previous two editions of *The Prokaryotes* (Bergey, 1981; Schroth et al., 1981; Schroth et al., 1992; Stolp and Gadkari, 1981; Palleroni, 1992) were published. The heterogeneity of *Pseudomonas* has been well documented from comprehensive studies dating to more than 40 years ago. For example, the detailed survey by Stanier et al. (1966) of 267 strains of aerobic pseudomonads clearly exposed the catabolic diversity of the species comprising the genus and provided insight into the phylogenetic diversity that would become more apparent a few years later. Much of what can be written today about *Pseudomonas*, particularly related to the phenotypic, as well as metabolic characteristics, has already been described in detail by several different groups (Clark and Ornston, 1975; Clark and Richmond, 1975; Palleroni, 1975).

Wherever possible, the focus of this compilation on nonmedical aspects of the genus *Pseudomonas* is directed at information that has come to light since the last edition of *The*

*Prokaryotes* in 1992. Much of microbial systematics has “evolved” in new directions within the last decade as a result of being able to access the phylogenetic relationships of microbial taxa. Elucidation of the “natural” relationships of bacteria has had a profound impact on the systematic reorganization of bacteria, in general, and on *Pseudomonas*, in particular. Many of the species that were described previously as *Pseudomonas* have been reclassified within new genera, and recognition of the phylogenetic heterogeneity of bacteria classified as *Pseudomonas* has initiated re-evaluations of the phenotypic characteristics, metabolic activities, genetics, ecology and other characteristics, in light of the inter- and intrageneric phylogenetic relationships. The early studies on the phylogenetic analyses of *Pseudomonas*, in fact, helped develop much of the methodology and technique that would be applied to the analyses of other prokaryotes in the years to come. Recently, advances in molecular biology, genome sequencing (the genomes of four species of *Pseudomonas* have been determined at the time of this writing), genomics and proteomics have presented a wealth of data for “pseudomonadologists” to access in trying to understand all facets of these bacteria. Researchers have only recently started to scale the mountain of information available.

### Phylogeny

The phylogenetic heterogeneity of pseudomonads was recognized initially from work on the comparative biochemistry of the bacteria (Stanier, 1968), which established the groundwork upon which much of the later work was based. The ability to further differentiate *Pseudomonas* (sensu stricto) from phenotypically similar bacteria has been due, primarily, to the development and application of methods for analyzing bacteria at the molecular level. Palleroni and colleagues at the University of California at Berkeley (Palleroni et al., 1973) were able to discern distinct intrageneric



groupings among, what were, at that time, the species comprising the genus *Pseudomonas*, by the use of rRNA similarities. The group of De Ley, at the Universiteit Gent in Belgium, also applied the use of rRNA-DNA hybridization to an extensive number of studies on the pseudomonads. De Vos et al. (1989) proposed, and it is now generally accepted, that the genus *Pseudomonas* is limited to the species related to *P. aeruginosa* in the DNA-rRNA homology group I (Palleroni et al., 1973), within the  $\gamma$ -subclass of the Proteobacteria (Woese et al., 1985), currently reorganized as the class "Gammaproteobacteria" (Krieg and Garrity, 2001). The species comprising this grouping have been referred to as the "true *Pseudomonas*" or the "fluorescent pseudomonads" because of the notable fluorescent, water-soluble pigments produced by *P. aeruginosa*, *P. fluorescens* and some other well-known species of the genus. Nevertheless, to add yet another note of confusion to the complexity of *Pseudomonas*, not all species of the so-called "fluorescent pseudomonads" actually produce fluorescent pigments, e.g., *P. alcaligenes*, *P. mendocina*, *P. stutzeri*, to name but a few. To differentiate systematically *Pseudomonas* (sensu stricto) from other bacteria that were included previously in the genus or those that are newly isolated and characterized, analyses of ribosomal RNA (rRNA) similarities have proven applicable for inferring definitive systematic relationships to the genus level (in most cases) and to subgenus levels (in some cases).

The rRNA sequence similarities between *Pseudomonas* species were determined initially by hybridization of DNA to ribosomal rRNA, in the group of Roger Stanier at the University of California at Berkeley (Palleroni, et al., 1973). The hybridization methodology revealed internal subdivisions of five distinct rRNA "homology groups" that corresponded to the levels of differentiation observed between different genera or families. The observed subdivisions of *Pseudomonas* were confirmed repeatedly by various methodologies performed throughout the following years in many other laboratories (Byng et al., 1983; De Vos and De Ley, 1983; Oyaizu and Komagata, 1983; De Vos et al., 1985; De Vos et al., 1989; Stead, 1992).

Subsequent to the analyses using the DNA-rRNA hybridization methods, another approach targeting rRNAs was applied to the analysis of *Pseudomonas* in the laboratory of Carl Woese at the University of Illinois. Utilizing a protocol of sequence determination and profiling of rRNA oligonucleotides to produce taxa-specific "catalogues" (Fox et al., 1980), Woese et al. (1984) described the diversity of the pseudomonads along the same divisions that had been recognized previously by the Berkeley group.

Essentially, some species that were classified as members of the genus *Pseudomonas* were observed to be less similar phylogenetically to other pseudomonads than they were to non-pseudomonad bacteria, e.g., enterics, phototrophs, nitrogen-fixing plant symbionts, etc. With such approaches, the "natural" system of bacterial classification that had been proposed as theoretically feasible by microbiologists such as Van Niel (1946), but which had remained so elusive practically, was acknowledged to offer significant possibilities for bacterial systematics.

While the DNA-rRNA hybridization and rRNA oligonucleotide cataloguing methods were able to provide overviews of the phylogenetic relationships of bacteria, the proponents of the methods acknowledged their respective limitations in being able to differentiate the closely related organisms (Lane et al., 1985; Woese, 1987). To this end, complete sequence determinations of the rRNAs were envisioned as the means for establishing a phylogeny-based bacterial systematics with the resolution to differentiate even the most closely related organisms. The polymerase chain reaction (PCR; Mullis and Faloona, 1987), combined with advances in DNA sequencing (Hunkapiller et al., 1991), has enabled the determination and comparison of rRNA gene sequences (i.e., rDNA) to become a practical methodology in most microbiology laboratories for rapidly analyzing large numbers of organisms (Edwards et al., 1989). Thus, the number of prokaryotic small subunit (i.e., 16S) rRNA/rDNA sequences compiled in databases has grown from less than 300 in 1990 (Neefs et al., 1991) to more than 35,000 (nearly full-length), as of June 2004 see the Ribosomal Database Project - II (<http://rdp.cme.msu.edu>) (Cole et al., 2003). Of these, only five pseudomonad 16S rRNA sequences were available in 1990. Currently, more than 1300 16S rRNA/rDNA sequences of strains of *Pseudomonas* species are available in the public databases.

The differentiation and inferred phylogenetic relationships of the various pseudomonad rRNA similarity groups, as well as of the species included in each one of the groups, by comparative analysis of 16S rRNA gene sequences has been described in detail in two comprehensive reviews (Kerstens et al., 1996; Anzai et al., 2000). Not surprisingly, the phylogenetic relationships inferred from 16S rDNA sequence analyses corresponded with the earlier results determined by rRNA-DNA hybridization and rRNA cataloguing data. Thus, species retained within the genus *Pseudomonas* (sensu stricto) are observed to be those related to *P. aeruginosa*, in the Gammaproteobacteria, while other species are seen to cluster within genera of the Alphaproteobacteria

and Betaproteobacteria. The bacteria most closely related to the genus *Pseudomonas* include the species of the aerobic, free-living, nitrogen-fixing *Azotobacter*-*Azomonas* complex, cellulolytic species of the genus *Cellvibrio* and, somewhat more distantly related, marine bacteria of the genera *Microbulbifer* and *Marinobacterium*, endosymbiotic bacteria of the genus *Teredinibacter*, halophilic bacteria of the genera *Halomonas*, *Oceanospirillum* and *Marinomonas*, *Marinobacter* species isolated from marine environments, the Moraxellaceae family, and methylotrophs of the Methylococcaceae (Kerstens et al., 1996; Anzai et al., 2000). These organisms exhibit quite diverse phenotypes and, on the basis of traditional analyses of phenotypic characteristics, probably would not be suspected as being the evolutionary “cousins” of *Pseudomonas*.

## Taxonomy

The genus *Pseudomonas* comprises Genus I of the bacterial family Pseudomonadaceae. Five genera (*Pseudomonas*, *Azotobacter*, *Azomonas*, *Azorhizophilus* and *Cellvibrio*) are assigned to the family. Common to all constituent genera are certain physiological properties, such as aerobic, chemoorganotrophic metabolism, absence of fermentation, absence of photosynthesis, and capacity for growth at the expense of a large variety of organic substrates. There are a few exceptions to these general properties, but these phenotypic criteria are generally common to all members of the family.

The original creation of the genus *Pseudomonas* established a taxon based solely upon characteristics of cell morphology. At this point, it is appropriate to point out a discrepancy with respect to the publication date for the presentation of the genus. While the date for the publication of the genus *Pseudomonas* has been recognized as being 1894, Gunsalus (1996) and Zumft (1997) have pointed out previously that the genus was, in fact, presented for the first time in 1895 by Walter Migula at the Bacteriologischen Institut der Technischen Hochschule zu Karlsruhe in his publication of a seven-year effort, “Ueber ein neues System der Bakterien” (Migula, 1895), to describe and compare all known bacteria. The initial description of *Pseudomonas* by Migula was based solely upon morphological characteristics, as follows (translation):

Genus *Pseudomonas* nov. gen.

Cells with polar flagella. Endospore formation occurs in some species, but infrequently (e.g., *Pseudomonas violacea*).

This “succinct” description established the new genus within the family Bacteriaceae, accommodating bacteria on the basis of characteristic flagella type and the greenish or blue-green fluorescent pigment of the pus collected in bandages of hospital patients and wounded soldiers. A more comprehensive description of the genus followed in 1900 (Migula, 1900), including 75 species and registering *Pseudomonas aeruginosa*, previously described as “*Bacterium aeruginosum*” (Schroeter, 1872), as the type species of the genus.

Winslow et al. (1917) established the family Pseudomonadaceae, encompassing the genus *Pseudomonas* and a number of other genera, many of which have been reclassified throughout the following years. Many of the species of genera that were categorized within the family Pseudomonadaceae have come to be regarded as “pseudomonads” and, by association, related to *Pseudomonas*. This has proven often to be a source of confusion. Two terms should be defined at this point to clarify an important nomenclatural distinction that is often confusing. “*Pseudomonas*” (capitalized and written in italics) is the validly published (i.e., with nomenclatural standing) name of a bacterial genus comprising species of defined collective phenotypic characteristics. On the other hand, “pseudomonad” (not capitalized and not italicized) is a descriptive term (i.e., *Pseudomonas*-like), with no formal nomenclatural status, accorded to a nonexclusive collection of bacteria exhibiting various levels of similarity to species of the genus *Pseudomonas*. Pseudomonad bacteria previously characterized according to a limited selection of traits may have been observed to be similar to bacteria previously identified (correctly or incorrectly) as *Pseudomonas* species. Unfortunately, taxonomic convention, combined with a degree of uncertainty associated with the identifications of some pseudomonad bacteria, has led to the point that the terms “*Pseudomonas*” and “pseudomonad” are sometimes used interchangeably and incorrectly.

In the eighth edition of *Bergey’s Manual of Determinative Bacteriology*, 29 well-characterized species of *Pseudomonas* were listed, with another 206 less well-described species included as addenda (Douderoff and Palleroni, 1974a). This was the first compilation of the Bergey’s series that incorporated molecular data, i.e., the G+C content of genomic DNA (Marmur and Doty, 1962), in the descriptions of the bacterial species. However, the level of taxonomic resolution that genomic DNA mol% G+C could afford was limited and, at that time, the data were handled simply as an additional determinative characteristic without any systematic weight.

The Approved Lists of Bacterial Names published in the *International Journal of Systematic Bacteriology* in 1980 (Skerman et al., 1980), provided an inventory of bacterial species names described before 1980 that were recognized as having formal nomenclatural standing under the auspices of the International Code of Nomenclature of Bacteria (1976 Revision). The list included 87 *Pseudomonas* species, effectively reducing the number of *Pseudomonas* nomen-species by not including names that were inconsistent with the rules of the Code.

In 1984, in the first edition of *Bergey's Manual of Systematic Bacteriology*, a listing of 94 *Pseudomonas* nomen-species was presented (Palleroni, 1984b). Many of the species were included with the recognition that their taxonomic relationships were unclear but with the expectation that comprehensive analyses would conclusively define their taxonomic positions. Within the first edition of *Bergey's Manual of Systematic Bacteriology*, perhaps the most significant contribution to the taxonomy of *Pseudomonas* was the presentation of the subdivision of the genus on the basis of rRNA similarities and estimations of phylogenetic relatedness (Palleroni et al., 1973; Palleroni, 1984). The five distinct "rRNA homology" groups were: 1) rRNA group I—*P. aeruginosa*, *P. fluorescens*, *P. putida* and related species, also termed the "true" *Pseudomonas* (i.e., *Pseudomonas sensu stricto*); this group included species observed to cluster within Woese's  $\gamma$ -subdivision of the Proteobacteria (Woese et al., 1985; Stackebrandt et al., 1988); 2) rRNA group II—*P. cepacia*, *P. mallei* and related species (to be reclassified as *Burkholderia*) and *P. solanacearum*, *P. picketti* and related species (to be reclassified as *Burkholderia* and, subsequently, as *Ralstonia*); this group included species observed to cluster within Woese's  $\beta$ -subdivision of the Proteobacteria (Woese et al., 1984); 3) rRNA group III—*P. testosteroni* and related species (to be reclassified as *Comamonas*), *P. acidovorans* and related species (to be reclassified as *Comamonas* and, subsequently, as *Delftia*), *P. facilis* and related species (to be reclassified as *Acidovorax*), *P. palleronii* and related species (to be reclassified as *Hydrogenophaga*), and *P. saccharophila* (not reclassified, to date); this group included species also observed to cluster within Woese's  $\beta$ -subdivision of the Proteobacteria; 4) rRNA group IV—*P. diminuta* and *P. vesicularis* (to be reclassified as *Brevundimonas*); this group included species observed to cluster within Woese's  $\alpha$ -subdivision of the Proteobacteria (Woese et al., 1984); and 5) rRNA group V—*P. maltophilia* (reclassified as *Xanthomonas* and, subsequently, as *Stenotrophomonas*); this group included species observed to cluster also within Woese's  $\gamma$ -subdivision of

the Proteobacteria but clearly distinct from *Pseudomonas*.

These data effectively established the framework for the modern taxonomy of *Pseudomonas*, as well as other pseudomonads. Since then, many of the organisms described initially as species of *Pseudomonas* have been reclassified and new species have been added to the genus, largely because of the ability to recognize the phylogenetic relationships that have been determined through genotypic characterization. *Pseudomonas*, described in the second edition of *Bergey's Manual of Systematic Bacteriology* (Palleroni, 2004), comprises 61 species, representing the number of species of the genus that were available in the literature until the end of year 2000. Since the beginning of year 2001 until the time of this writing (June 2004), 30 new species of *Pseudomonas* have been described and the names validly published.

The taxonomic status of *Pseudomonas*, including the reclassifications of species formerly included within the genus, and the addition of new species, can be observed on-line through the internet at two principal sites: 1) the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) site for "Bacterial Nomenclature Up-to-Date" (<http://www.dsmz.de/bactnom/bactname.htm>); and 2) the (<http://www.bacterio.cict.fr>{site}) of the École Nationale Vétérinaire, "J.P. Euzéby "List of Bacterial Names with Standing in Nomenclature." These sites have compiled all the bacterial names of the "Approved Lists of Bacterial Names" (Skerman et al., 1980), as well as those that have been validly published since January 1, 1980.

Currently, *Pseudomonas* includes 156 species names that are recognized with nomenclatural standing, although 48 of these species have been reclassified and are now considered to be basonyms or synonyms of species placed in other genera (Table 1). Two species, *P. aureofaciens* and *P. perfectomarina*, are recognized as later heterotrophic synonyms of *P. chlororaphis* and *P. stutzeri* (genomovar 2), respectively. Another 10 species are recognized as not belonging to *Pseudomonas* (*sensu stricto*) but have not been reclassified as yet. At the time of this writing, 96 species have been described and recognized as belonging to the genus *Pseudomonas* (*sensu stricto*) and are listed with names of nomenclatural standing.

## Habitat

*Pseudomonas* is a genus of truly ubiquitous organisms, which seems to be a consequence of their simple nutritional requirements, the range

Table 1. *Pseudomonas* species with validly published names and their current taxonomic status.

Species name <sup>a</sup>	Type strain	Re-classification <sup>b</sup>	16S rDNA accession no. <sup>c</sup>
<i>P. abietaniphila</i>	ATCC 700689		AJ011504
<i>P. acidovorans</i> <sup>d</sup>	DSM 39	<i>Delftia acidovorans</i>	
<i>P. aeruginosa</i> <sup>e</sup>	LMG 1242		Z76651
<i>P. agarici</i>	LMG 2112		Z76652
<i>P. alcaligenes</i>	LMG 1224		Z76653
<i>P. alcaliphila</i>	JCM 10630		AB030583
<i>P. aminovorans</i> <sup>d</sup>	DSM 7048	<i>Aminobacter aminovorans</i>	
<i>P. amygdali</i>	LMG 1223		Z76654
<i>P. andropogonis</i> <sup>d</sup>	DSM 9511	<i>Burkholderia andropogonis</i>	
<i>P. anguilliseptica</i>	NCIMB 1949		AB021376
<i>P. antarctica</i>	DSM 15318		AJ537601
<i>P. antimicrobica</i> <sup>d</sup>	LMG 18920	<i>Burkholderia gladioli</i>	
<i>P. asplenii</i>	LMG 2137		Z76655
<i>P. aurantiaca</i>	ATCC 33663		AB021412
<i>P. aureofaciens</i> <sup>f</sup>	DSM 6698	<i>P. chlororaphis</i>	Z76656
<i>P. avellanae</i>	DSM 11809		
<i>P. avenae</i> <sup>d</sup>	DSM 7227	<i>Acidovorax avenae</i>	
<i>P. azotoformans</i>	IAM 1603		D84009
<i>P. balearica</i>	DSM 6083		U26418
<i>P. beijerinckii</i> <sup>d</sup>	DSM 7218	( <i>Halobacter</i> sp.)	
<i>P. beteli</i> <sup>d</sup>	LMG 978	( <i>Stenotrophomonas</i> sp.)	
<i>P. boreopolis</i> <sup>d</sup>	LMG 979	( <i>Xanthomonas</i> sp.)	
<i>P. brassicacearum</i>	CFBP 11706		AJ293858
<i>P. brenneri</i>	CIP 106646		AF268968
<i>P. cannabina</i>	CFBP 2341		AJ492827
<i>P. carboxydohydrogena</i> <sup>d</sup>	DSM 1083		
<i>P. caricapapayae</i>	ATCC 33615		D84010
<i>P. caryophylli</i> <sup>d</sup>	JCM 9310	<i>Burkholderia caryophylli</i>	
<i>P. cattleyae</i> <sup>d</sup>	LMG 5286	<i>Acidovorax avenae</i>	
<i>P. cedrina</i>	CIP 105541		AF064461
<i>P. cepacia</i> <sup>d</sup>	LMG 1222	<i>Burkholderia cepacia</i>	
<i>P. chloritidismutans</i>	DSM 13592		AZ017341
<i>P. chlororaphis</i>	LMG 5004		Z76657
<i>P. cichorii</i>	LMG 2162		Z76658
<i>P. cissicola</i> <sup>d</sup>	LMG 2167	( <i>Xanthomonas</i> sp.)	
<i>P. citronellolis</i>	DSM 50332		Z76659
<i>P. cocovenenans</i> <sup>d</sup>	DSM 11318	<i>Burkholderia cocovenenans</i>	
<i>P. congelans</i>	DSM 14939		AJ492828
<i>P. corrugata</i>	ATCC 29736		D84012
<i>P. constantinii</i>	CFBP 5705		AF374472
<i>P. cremoricolorata</i>	IAM 1541		AB060137
<i>P. delafieldii</i> <sup>d</sup>	DSM 64	<i>Acidovorax delafieldii</i>	
<i>P. denitrificans</i> <sup>d</sup>	ATCC 19244	<i>nomen ambiguum</i>	
<i>P. diminuta</i>	DSM 7234	<i>Brevundimonas diminuta</i>	
<i>P. doudoroffii</i> <sup>d</sup>	DSM 7028	<i>Oceanimonas doudoroffii</i>	
<i>P. echinoides</i> <sup>d</sup>	DSM 50409	<i>Sphingomonas echinoides</i>	
<i>P. elongata</i> <sup>d</sup>	DSM 6810	<i>Microbulbifer elongatus</i>	
<i>P. extremorientalis</i>	LMG 19695		AF405328
<i>Pseudomonas facilis</i> <sup>d</sup>	DSM 649	<i>Acidovorax facilis</i>	
<i>P. ficuserectae</i> <sup>f</sup>	LMG 5694	( <i>P. amygdali</i> )	Z76661
<i>P. flava</i> <sup>d</sup>	DSM 619	<i>Hydrogenophaga flava</i>	
<i>P. flavescens</i>	ATCC 51555		U01916
<i>P. flectens</i> <sup>d</sup>	LMG 2187		
<i>P. fluorescens</i>	DSM 50090		Z76662
<i>P. fragi</i>	IFO 3458		AB021413
<i>P. frederiksbergensis</i>	DSM 13022		AJ249382
<i>P. fulva</i>	IAM 1529		D84015
<i>P. fuscovaginae</i> <sup>f</sup>	MAFF 301177	( <i>P. fuscivaginae</i> )	AB021381
<i>P. gelidicola</i>	IAM 1127		
<i>P. geniculata</i> <sup>d</sup>	LMG 2195	( <i>Stenotrophomonas</i> sp.)	
<i>P. gessardii</i>	CIP 105469		AF074384
<i>P. gladioli</i> <sup>d</sup>	DSM 4285	<i>Burkholderia gladioli</i>	

Table 1. *Continued*

Species name <sup>a</sup>	Type strain	Re-classification <sup>b</sup>	16S rDNA accession no. <sup>c</sup>
<i>P. glathei</i> <sup>d</sup>	DSM 50014	<i>Burkholderia glathei</i>	
<i>P. glumae</i> <sup>d</sup>	DSM 9512	<i>Burkholderia glumae</i>	
<i>P. graminis</i>	DSM 11363		Y11150
<i>P. grimontii</i>	CIP 106645		AF268029
<i>P. halophila</i>	DSM 3050		AB021383
<i>P. hibiscicola</i> <sup>d</sup>	LMG 980	( <i>Stenotrophomonas</i> sp.)	
<i>P. huttiensis</i> <sup>d</sup>	DSM 10281	( <i>Herbaspirillum</i> sp.)	
<i>P. indica</i>	DSM 14015		AF302795
<i>P. indigofera</i> <sup>d</sup>	DSM 3303	<i>Vogesella indigofera</i>	
<i>P. iners</i> <sup>d</sup>	CIP 106746	<i>Marinobacterium georgiense</i>	
<i>P. jessenii</i>	CIP 105274		AF06825
<i>P. jinjuensis</i>	LMG 21316		AF468448
<i>P. kilonensis</i>	DSM 13647		AJ292426
<i>P. koreensis</i>	LMG 21318		AF468452
<i>P. lanceolata</i>	ATCC 14669		AB021390
<i>P. lemoignei</i> <sup>d</sup>	DSM 7445	<i>Paucimonas lemoignei</i>	
<i>P. libanensis</i>	CIP 105460		AF057645
<i>P. lini</i>	ICMP 14138		AY035996
<i>P. lundensis</i>	ATCC 49968		AB021395
<i>P. lutea</i>	LMG 21974		AY364537
<i>P. luteola</i>	IAM 13000		D84002
<i>P. mallei</i> <sup>d</sup>	ATCC 23344	<i>Burkholderia mallei</i>	
<i>P. maltophilia</i> <sup>d</sup>	DSM 50170	<i>Stenotrophomonas maltophilia</i>	
<i>P. mandelii</i>	CIP 105273		AF058286
<i>P. marginalis</i>	LMG 2210		Z76663
<i>P. marina</i> <sup>d</sup>	DSM 4741	<i>Cobetia marina</i>	
<i>P. mediterranea</i>	CFBP 5447		AF386080
<i>P. meliae</i> <sup>f</sup>	LMG 2220	( <i>P. amygdali</i> )	AB021382
<i>P. mendocina</i>	LMG 1223		Z76664
<i>P. mephitica</i>	ATCC 33665		AB021388
<i>P. meridiana</i>	DSM 15319		AJ537602
<i>P. mesophilica</i> <sup>d</sup>	DSM 1708	<i>Methylobacterium mesophilicum</i>	
<i>P. migulae</i>	CIP 105470		AF074383
<i>P. mixta</i> <sup>d</sup>	DSM 4832	<i>Telluria mixta</i>	
<i>P. montelii</i>	CIP 104883		AB021409
<i>P. mosselii</i>	CIP 105259		AF072688
<i>P. mucidolens</i>	IAM 12406		D84017
<i>P. multiresivorans</i>	ATCC 700690		X96787
<i>P. nautica</i> <sup>d</sup>	DSM 50418	<i>Marinobacter hydrocarbonoclasticus</i>	
<i>P. nitroreducens</i>	IAM 1439		D84021
<i>P. oleovorans</i>	DSM 1045		Z76665
<i>P. orientalis</i>	CIP 105540		AF064457
<i>P. oryzihabitans</i>	IAM 1568		D84004
<i>P. palleroniana</i>	CFBP 4389		AY091527
<i>P. palleronii</i> <sup>d</sup>	DSM 63	<i>Hydrogenophaga palleronii</i>	
<i>P. parafulva</i>	JCM 11244		AB06013
<i>P. paucimobilis</i> <sup>d</sup>	DSM 1098	<i>Sphingomonas paucimobilis</i>	
<i>P. perfectomarina</i> <sup>f</sup>	ATCC 14405	<i>P. stutzeri</i>	
<i>P. pertucinogena</i>	IFO 14163		AB021380
<i>P. phenazinium</i> <sup>d</sup>	DSM 10684	<i>Burkholderia phenazinium</i>	
<i>P. picketti</i> <sup>d</sup>	DSM 6297	<i>Ralstonia pickettii</i>	
<i>P. pictorum</i> <sup>d</sup>	LMG 981	( <i>Stenotrophomonas</i> sp.)	
<i>P. plantarii</i> <sup>d</sup>	DSM 9509	<i>Burkholderia plantarii</i>	
<i>P. plecoglossicida</i>	DSM 15088		AB009457
<i>P. poae</i>	DSM 14936		AJ492829
<i>P. proteolytica</i>	DSM 15321		AJ537603
<i>P. pseudoalcaligenes</i>	LMG 1225		Z76666
<i>P. pseudoflava</i> <sup>d</sup>	DSM 1034	<i>Hydrogenophaga pseudoflava</i>	
<i>P. pseudomallei</i> <sup>d</sup>	ATCC 23343	<i>Burkholderia pseudomallei</i>	
<i>P. psychrophila</i>	JCM 10889		AB041885
<i>P. putida</i>	DSM 291		Z76667
<i>P. pyrrocinia</i> <sup>d</sup>	DSM 10685	<i>Burkholderia pyrrocinia</i>	
<i>P. radiora</i> <sup>d</sup>	DSM 1819	<i>Methylobacterium radiotolerans</i>	

Table 1. *Pseudomonas*

Species name <sup>a</sup>	Type strain	Re-classification <sup>b</sup>	16S rDNA accession no. <sup>c</sup>
<i>P. resinovorans</i>	LMG 2274		Z76668
<i>P. rhizosphaerae</i>	LMG 21640		AY152673
<i>P. rhodesiae</i>	CIP 104664		AB021410
<i>P. rhodos</i> <sup>d</sup>	DSM 2163	<i>Methylobacterium rhodinum</i>	
<i>P. rubrilineans</i> <sup>d</sup>	LMG 2281	<i>Acidovorax avenae</i>	
<i>P. rubrisubalbicans</i> <sup>d</sup>	DSM 11543	<i>Herbaspirillum rubrisubalbicans</i>	
<i>P. saccharophila</i> <sup>d</sup>	DSM 654		
<i>P. salomonii</i>	CFBP 2022		AY091528
<i>P. savastanoi</i> <sup>f</sup>	ATCC 13522	( <i>P. amygdali</i> )	AB021402
<i>P. solanacearum</i> <sup>d</sup>	DSM 9544	<i>Ralstonia solanacearum</i>	
<i>P. spinosa</i>	ATCC 14606		AB021387
<i>P. stanieri</i> <sup>d</sup>	DSM 7027	<i>Marinobacterium stanieri</i>	
<i>P. straminea</i>	IAM 1598		D84023
<i>P. stutzeri</i>	CCUG 11256		U26262
<i>P. synxantha</i>	IAM 12356		D84025
<i>P. syringae</i>	LMG 1247		Z76669
<i>P. syzygii</i> <sup>d</sup>	DSM 7385	<i>Ralstonia syzygii</i>	
<i>P. taeniospiralis</i> <sup>d</sup>	DSM 2082	<i>Hydrogenophaga taeniospiralis</i>	
<i>P. taetrolens</i>	IAM 1653		D84026
<i>P. testosteroni</i> <sup>d</sup>	DSM 50244	<i>Comamonas testosteroni</i>	
<i>P. thermotolerans</i>	DSM 14292		AJ311980
<i>P. thivervalensis</i>	CFBP 11261		AF100323
<i>P. tolaasii</i>	LMG 2342		Z76670
<i>P. tremae</i>	CFBP 6111		AJ492826
<i>P. trivialis</i>	DSM 14937		AJ492831
<i>P. umsongensis</i>	KACC 10847		AF468450
<i>P. Vancouverensis</i>	ATCC 700688		AJ011507
<i>P. veronii</i>	CIP 104663		AB021411
<i>P. vesicularis</i> <sup>d</sup>	DSM 7226	<i>Brevundimonas vesicularis</i>	
<i>P. viridiflava</i>	LMG 2352		Z76671
<i>P. woodsii</i> <sup>d</sup>	LMG 2362	<i>Burkholderia andropogonis</i>	

Abbreviations: ATCC, American Type Culture Collection, Manassas, Virginia, United States; CFBP, Collection Francaise des Bacteries Phytopathogenes, Station de Pathologie Végétale et Phytobactériologie, Beaucouzé Dedex, France; CCUG, Culture Collection University of Göteborg, Göteborg, Sweden; CIP, Collection de l'Institut Pasteur, Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IAM, Institute for Applied Microbiology, University of Tokyo, Tokyo, Japan; ICMP, International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Hirowawa, Wako-shi, Japan; KACC, Korean Agricultural Culture Collection, National Institute of Agricultural Biotechnology, Seodun-dong Gwonseon-gu, Suwon, Korea; LMG, Laboratorium voor Mikrobiologie, Universiteit Gent, Gent, Belgium; and NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom.

<sup>a</sup>Validly published names of species of the genus *Pseudomonas*, species names that have not been validly published are not included.

<sup>b</sup>Reclassification names in parentheses indicate that a reclassification has not been formally proposed.

<sup>c</sup>Accession numbers are given only for species of *Pseudomonas* (sensu stricto).

<sup>d</sup>Species that should not be included within the genus *Pseudomonas*.

<sup>e</sup>*P. aeruginosa* is the type species of the genus.

<sup>f</sup>*Pseudomonas* species that should be reclassified with a new species name.

of carbon compounds they utilize, and their genetic and metabolic adaptability. *Pseudomonas* species have a wealth of habitats available to it, ranging from various soil and water environments to plant and animal tissue. Essentially, any habitat with a temperature range of 4–42°C, a pH between 4 and 8 and containing simple or complex organic compounds is a potential habitat for *Pseudomonas*. *Pseudomonas* species are aerobes and the requirement for oxygen is

apparently the major constraining factor for habitation by *Pseudomonas*.

In fact, *Pseudomonas* species are generally found in soils and water environments that have aerobic, mesophilic and neutral pH conditions. In nature, *Pseudomonas* species exist as saprophytes and parasites. In general, they are not prominent in anaerobic environments, and they do not occur in extreme thermophilic or acidophilic habitats. On the basis of the data

derived from cultivation-dependent analyses such as selective enrichments and isolations, *Pseudomonas* would appear to occupy a prominent position in nature.

The conditions under which *Pseudomonas* species survive in soil are also favorable for growth of aerobic actinomycetes of the genus *Streptomyces* (prominent in the degradation of complex organic compounds) as well as many other types of bacteria. Soil-dwelling *Pseudomonas* grow in association with the streptomycetes and other bacteria, which may be providing them with monomeric carbon sources for growth.

The phytopathogenic *Pseudomonas* generally can be found only on diseased plants, in which they appear as relatively homogeneous populations when the pathological lesions are young. The distribution of many of these pathogens outside their host plants is poorly known, although many seem to be able to exist as saprophytes, as well.

*Pseudomonas* species play important roles in the food industry, where spoilage of meats, poultry and fish occurs, even under refrigerated conditions (Barrett et al., 1986). Some species of *Pseudomonas* are found in tap water and in hospital saline solutions, which are presumably devoid of nutrients (Van der Krooij, 1977). *Pseudomonas* can be particularly problematic in the development of biofilms within pipelines. New species of *Pseudomonas* have recently been isolated from relatively oligotrophic mineral water sources (Elomari et al., 1996; Dabboussi et al., 1999; Verhille et al., 1999; Ivanova et al., 2002).

Bacteria, including *Pseudomonas* species, traditionally have been studied as “planktonic forms” in cultures of broth media. However, bacteria surviving in various natural ecosystems are observed to live in small or extensive biofilm environments (Tolker-Nielsen and Molin, 2004). Quorum-sensing appears to play important roles in the development of biofilms, through the sensing of population densities and the exertion of some control in the expression of genes involved in the various stages of biofilm development (Whiteley et al., 2001).

This section describes some of the various habitats and niches of *Pseudomonas* species. While this treatment is relatively general, recent reviews have presented much more detailed overviews of the soil, rhizosphere and phyllosphere habitats of *Pseudomonas* (Bailey, 2004; Lugtenberg and Bloemberg, 2004; Sørensen and Nybroe, 2004).

### *Pseudomonas* in Soil Environments

The soil environment may comprise the most complex of environmental niches that are

encountered by microorganisms. The heterogeneity of factors for growth in a soil presents the bacteria a challenging matrix of aggregate surfaces and pore structures. As the relationships between soil, plants and microbes are so intricately interwoven, it is difficult to obtain consistent overviews of the relevant microorganisms that are clearly demarcated between bulk soil and rhizosphere soil. *Pseudomonas* species, as well as species of many other pseudomonad genera, are clearly impacted by the rhizosphere influence.

Soil-dwelling *Pseudomonas* species are distributed throughout the world and, in general, may be observed to comprise similar rRNA sequence types and similar rRNA gene intergenic spacer (IS) types, as determined from a worldwide sampling of fluorescent species of *Pseudomonas* for assessing the inherent endemicity of microbial populations (Cho and Tiedje, 2000). However, at the level determined by genome fingerprinting (BOX-PCR) analyses, i.e., the strain level, *Pseudomonas* may be seen to comprise endemic populations that are determined by the specific transects of sampling sites.

A predominant question in microbial ecology is related to the actual presence and abundance of bacterial taxa within the respective ecosystems. Estimates suggest that cultivations are able to detect less than 1% of the total microbial populations in soil samples. Thus, while *Pseudomonas* species are able to be isolated from most soil samples, still unclear is whether the results obtained from enrichments and isolations reflect the situations in situ. In general, studies of various bulk soil ecosystems, employing cultivation-independent analyses of bacterial diversity, do not reflect the diversity or proportion of *Pseudomonas* species that are normally obtained by cultivation-dependent approaches. Even in xenobiotic-contaminated soils, which readily yield a diversity of isolates of *Pseudomonas* species, cultivation-independent approaches suggest that other bacterial taxa are numerically more predominant and that *Pseudomonas* may play only a minor role (Nogales et al., 1999; Nogales et al., 2001). However, such observations may not be contradicting the true nature of *Pseudomonas* species, as saprophytes and parasites. Even though *Pseudomonas*, as a group, may be observed to take advantage of its diverse metabolic potential and simple nutrient requirements, various *Pseudomonas* species, with their relatively rapid growth, may discover that they are less able to compete in bulk soil environments with other bacterial taxa that may be better adapted to dispersed levels of nutrients and that may possess various strategies for withstanding periods of “starvation”. While the soil ecosystem may appear to offer a diversity of

nutrient resources for microorganisms to use, the bioavailability of those resources, which may be complexed with inorganic matrices, also play an important role in selecting for which bacterial taxa are able to thrive in any given soil ecosystem. A comparative study of the bacterial taxa within bulk soil samples and in earthworm (*Lumbricus rubellus*) casts demonstrated significant differences in the numbers of *Pseudomonas* species detected, which was believed to be related to the nutrients available to the microorganisms during passage through the gut (Furlong et al., 2002). Limited available resources that may be obtained within bulk soil environments may not actually afford *Pseudomonas* species their best options and they may be more adapted to occupy an ecological niche as opportunistic scavengers in more nutrient-rich environments.

### *Pseudomonas* in Association with Plants

The rhizosphere is referred to as a “zone” influenced by plants (Hiltner, 1904). This zone around the roots of plants is one of intense microbial activity, due to the secretion of organic and amino acids by the plants. *Pseudomonas* species are among the most competent rhizosphere colonizers of soil (Lugtenberg et al., 2001). Lugtenberg and Bloemberg (2004) have described the traits necessary for successful rhizosphere colonization, i.e., motility and chemotaxis, specialized pili for attachment to surfaces, lipopolysaccharide (LPS) and outer membrane integrity for efficient uptake of nutrients, ability to synthesize vitamins and macromolecule building blocks, ability to utilize specific exudate components, resistance to toxins, and other plant defenses. *Pseudomonas* species, in general, possess all of these traits, which provide them with a selective advantage for exploiting the resources in the rhizosphere. *Pseudomonas* species also effectively inhibit the colonization of plants by other microorganisms (Bianciotto et al., 1996). Thus, *Pseudomonas* is seen as an important agent for biocontrol of plant diseases.

In addition to the microbial communities below ground, an extensive diversity of microorganisms (epiphytes) populate and interact with the surfaces of plants (phyllosphere), primarily the leaves. Bacteria that have adapted to life in the phyllosphere must possess characteristics that protect them from exposure, as well as nutrient and water limitations (Bailey, 2004). Mercier and Lindow (2000) have demonstrated the availability of amino acids, carbohydrates and organic acids that leach from the interior of the leaf to the exterior, providing significant levels of nutrients to plant surface colonizing

bacteria. Bacteria appear to exist on plant surfaces, predominantly, as biofilms (Morris et al., 1998).

Epiphytes, including *Pseudomonas* species, may affect plant productivity negatively, e.g., through induction of frost injury (Lindow, 1995), or positively, e.g., by production of phytohormones that enhance development (Brandi et al., 2001). Just as specific traits enable certain bacteria to colonize successfully the rhizosphere environment, traits have also been identified that are associated with epiphytic fitness. Some of these traits are correlated with locations on leaf surfaces of pathogenic (i.e., *P. syringae*) and non-pathogenic species, and with the ability of the pathogens to access and multiply in protected sites of the phyllosphere (Wilson et al., 1999).

The phytopathogenic species of *Pseudomonas* are diverse with respect to their genetics, ecology, and the diseases they cause. One species, *P. syringae*, includes more than 50 pathovars, most of which specifically colonize different plant hosts. Phytopathogenic *Pseudomonas* species are distributed worldwide, causing diseases of most major groups of higher plants. Besides the pathogenic associations, *Pseudomonas* species also exist in other types of associations with plants. Some species affect plant growth through their inhibition of fungal plant pathogens or by their effects on the roots of plants. *Pseudomonas* species may further colonize other plant parts, with no apparent damage or benefit to the plant (Lodewyckx et al., 2002).

## Isolation

*Pseudomonas* species, in general, have simple nutritional requirements and are readily isolated from a variety of environments. In the laboratory they grow well in media containing some organic matter in solution, at neutral pH, and at temperatures in the mesophilic range. The optimal growth temperature for *P. aeruginosa*, the most likely species to be encountered in medical specimens, is 37°C. However, species of *Pseudomonas* grow well at 28–30°C, which is more appropriate for most of the species.

The range of nutrient sources that are used by *Pseudomonas* is extensive and species of *Pseudomonas* tend to grow relatively rapidly, often outgrowing the species of other genera. Most species of *Pseudomonas* favor a rich medium, such as nutrient broth and agar or tryptic soy broth and agar and other media rich in peptides, although different numbers and diversity of *Pseudomonas* species may be obtained with different peptide concentrations in the media (Aagot et al., 2001). Saprophytic species of *Pseudomonas* can be isolated by streaking



samples on nutrient agar or tryptic soy agar plates. Denitrifying *Pseudomonas* species are isolated by specific enrichment procedures in a medium containing nitrate (NO<sub>3</sub>), under anaerobic conditions, at 30–40°C.

Most *Pseudomonas* species grow in chemically defined media without added growth factors. With the reorganizations of the taxonomy of *Pseudomonas*, species known to require special growth supplements are now recognized as belonging to separate and distinct evolutionary lineages. No particular minerals or vitamins are necessary for supporting growth of species of *Pseudomonas* (*sensu stricto*).

A medium described by Palleroni and Douderoff (1972), developed for autotrophic and heterotrophic enrichments, functions well for the cultivation of *Pseudomonas*. *Pseudomonas* species grow well also on mineral medium used for isolating *Arthrobacter* (Owens and Keddie, 1969) and on R2A mineral medium for oligotrophic growth (BBL Microbiology Systems, Cockeysville, MD). In general, good growth of *Pseudomonas* species is observed in media including organic compounds from 0.1–1.0% (w/v) as carbon and energy sources.

Cultivation media that have been used for selective enrichments of *Pseudomonas* species may be deficient in iron, enabling the detection of fluorescent *Pseudomonas* species. The fluorescence is due to increased production of siderophore pigments extruded into the media. Other media have employed antibiotics, such as penicillin G, novobiocin and cycloheximide, which do not inhibit fluorescent *Pseudomonas* species (Sands and Rovira, 1970).

Selective media used extensively for the detection and isolation of fluorescent *Pseudomonas* species are King's media A and B (King et al., 1954), which employ potassium and magnesium salts to enhance pyocyanin and pyoverdine pigment production. Gould's S1 medium contains sodium lauroyl sarcosine, which effectively inhibits the growth of Gram-positive bacteria. The antibiotic trimethoprim may be included in the medium to inhibit nonfluorescent pseudomonads (Fromin et al., 2001). Other selective-differential media that support *Pseudomonas* growth include Pseudosel agar medium (BBL Microbiology Systems), Cetrimide agar medium, *Pseudomonas* isolation agar medium and *Pseudomonas* agar F medium (Difco Laboratories, Detroit, MI) containing cetyltrimethylammonium bromide (Cetrimide or CTAB), 2,4,4-trichloro-2-hydroxydiphenyl ether (Irgasan), or similar compounds, although *Pseudomonas* species are isolated in lower numbers and diversity on the strongly selective media (Gilardi, 1985). The use of Cetrimide was recommended (Lowbury, 1951) for inhibiting the

growth of accompanying microbial flora and minimizing interference with the growth of *P. aeruginosa*. The pigment production of *P. aeruginosa* is not inhibited when grown on this medium. Addition of nalidixic acid (Goto and Enomoto, 1970) improves further the inhibition of accompanying microbial flora.

As Schroth et al. (1992) have pointed out, the choice between a general or selective medium for isolation of *Pseudomonas* depends upon the samples to be analyzed. Selective media are recommended for samples from soil, water and organic materials that contain many other microorganisms, whereas general growth media will be more useful for obtaining larger numbers and diversity of isolates from sources exhibiting strong selective pressures already.

Recently, new species of *Pseudomonas* have been isolated from various soil types (Delorme et al., 2002; Kwon et al., 2003), rhizosphere (Achouak et al., 2000), phyllosphere (Behrendt et al., 1999), water (Elomari et al., 1996; Dabboussi et al., 1999; Verhille et al., 1999; Ivanova et al., 2002), and Antarctic cyanobacterial mats (Gundlapalli et al., 2004), as well as by the application of some uncommon selection protocols, including, the use of resin acids, which may be biologically toxic (Mohn et al., 1999), chlorate as a terminal electron acceptor and acetate as an electron donor (Wolterink et al., 2002), and cooking water from a cork-processing factory at 50°C (Manaia and Moore, 2002).

## Identification

Straight or slightly curved rods but not helical, 0.5–1.0 µm in diameter by 1.5–5.0 µm in length. Most of the species do not accumulate granules of poly-β-hydroxybutyrate, but accumulation of poly-hydroxyalkanoates of monomer lengths higher than C<sub>4</sub> may occur when growing on alkanes or gluconate. Do not produce prosthecae and are not surrounded by sheaths. No resting stages are known. Gram-negative. Motile by one or several polar flagella; rarely nonmotile. In some species, lateral flagella of short wavelength may also be formed. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor; in some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. Xanthomonadins are not produced. Most, if not all, species fail to grow under acid conditions (pH 4.5 or lower). Most species do not require organic growth factors. Oxidase-positive or negative. Catalase-positive. Chemoorganotrophic. Strains of the species include in their composition the hydroxylated fatty acids 3-OH 10:0 and 12:0, and 2-OH 12:0, and ubiquinone Q-9.

Widely distributed in nature. Some species are pathogenic for humans, animals or plants. The mol% G+C content of the DNA is 58–69. Type species: *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900, 884.

The above is the definition of *Pseudomonas*, as it appears in the second edition of *Bergey's Manual of Systematic Bacteriology* (Palleroni, 2005). Characterizations of bacteria, including *Pseudomonas*, must address at least two aspects for purposes of identification. The first has to do with the characterization of *Pseudomonas* (sensu stricto) and differentiation from other genera, including those species that were classified as *Pseudomonas* in the past. The second has to do with resolving the intrageneric subdivisions of *Pseudomonas* (sensu stricto) into species. A complicating factor for such analyses is that species of the genus *Pseudomonas* and other pseudomonads are well known for their “promiscuity” in exchanging to exchanging genetic material. Questions of whether observed traits are stable, reliable and truly characteristic of a given organism become relevant for taxonomic and identification purposes.

The “species” is the basic unit of classification in biology and, as well, has been applied in microbiology, albeit relatively inconsistently. The intrageneric units in microbiology have been accepted somewhat empirically and guidelines for determining what is necessary for describing a bacterial species in any systematic manner have only recently evolved. Rosselló-Mora and Amann (2001) have discussed the complexities of conceptualizing the prokaryotic species, and have proposed guidelines for categorizing what they have termed the “phylo-phenetic species,” i.e., “a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property.”

The genus *Pseudomonas* comprises a group of species for which the amount of information available is quite uneven. It is relatively vast in the case of the type species of the genus, *P. aeruginosa*, primarily because of its medical importance as an opportunistic pathogen. However, markedly less is known about other species of the genus, particularly those isolated recently from some relatively uncommon sources. Perhaps, owing to better recognition of the properties useful in determinative schemes, the isolation of bacteria, such as *P. mendocina*, *P. stutzeri* and other *Pseudomonas* species, are being reported with increasing frequency from clinical samples. Generally speaking, *Pseudomonas* has a well-deserved reputation for a variety of beneficial biotechnological properties. In the case of *P. fluorescens* and *P. putida*, catabolic

properties have dominated other details of *Pseudomonas* biology, including the recently growing amount of information on the effect of these species on stimulating plant growth. *P. fluorescens* and *P. putida* are represented in nature by several biovars, and the species names lack precise systematic definition. However, the genus also is notorious because of species that are opportunistic pathogens of humans as well as phytopathogens that significantly impact the agricultural and economic well-being worldwide.

While some species of the genus *Pseudomonas* (e.g., *P. aeruginosa*) are homogeneous taxonomic units that are relatively easily differentiated and identified, other species include complex subdivisions, such as the *P. fluorescens* and *P. putida* biovars, the *P. syringae* and *P. marginalis* pathovars, or the *P. stutzeri* genomovars. Such internal organization of the genus is a reflection of the heterogeneity of bacteria that have been studied extensively and whose characteristics have not conformed completely to recognized taxonomy. Discrepancies between phenotypic and genotypic properties have further hindered attempts to resolve the systematics. However, given these complications in bacterial taxonomy, the “species” remains the biological unit that purports to represent a systematic description of an organism's basic properties.

Traditional and more recent characteristics applicable to *Pseudomonas* identification at the genus and subgenus levels are described below.

### Cell Morphology

*Pseudomonas* species are straight or slightly curved rod-shaped cells (maximum length ca. 4.0  $\mu\text{m}$ ) that occur singly or in pairs or short chains. Their morphology may occasionally deviate from that in standard descriptions. The cells typically stain Gram-negative. Electron microscopy reveals cell walls and membranes typical of Gram-negative bacteria, although different species may exhibit different chemical compositions. The cells of many of the species lyse readily in EDTA solution, which correlates generally with the high phosphorous content of the outer membranes (Wilkinson, 1970). Many species of *Pseudomonas* have been observed to produce poly- $\beta$ -hydroxybutyrate (PHB) granules, particularly when grown under nitrogen-limiting conditions. The traditional description of a short rod-shaped cell does not always fit the morphology of all strains of *Pseudomonas* species. The cells of some strains can be extremely short, while in others (*P. putida* and *P. syringae*) they may be unusually long. Particularly in the cases of older cultures, the cells may be of unusual shapes and sizes.

With few exceptions, flagellar motility is a common characteristic of *Pseudomonas* species. Motility allows the cells to respond to chemical stimuli (chemotaxis) and enhances the ability of the bacterium to locate organic substrates at low concentrations. The majority of *P. aeruginosa* cells have only one flagellum, although exceptional *P. aeruginosa* cells with two or three flagella have been observed. *P. alcaligenes*, *P. mendocina*, *P. pseudoalcaligenes* and *P. stutzeri* are also characterized by having a single flagellum per cell. Most other species possess more than one. However, variations are observed in the flagellation of strains of *Pseudomonas* species and nonmotile strains may be isolated occasionally. The flagellar number of *P. aeruginosa* is reported to be controlled by FleN, a putative ATP-GTP binding protein, and the disruption of the *fleN* gene results in multiflagellation of otherwise monoflagellated strains, as well as in chemotactic defects (Dasgupta et al., 2000). The flagella are inserted at the poles of cells, although some exceptions have been observed, e.g., in *P. stutzeri* (Palleroni et al., 1970). Lateral flagella are thought to participate in swarming activity of cells (Shinoda and Okamoto, 1977).

Pili are observed in many Gram-negative bacteria, functioning as appendages for attaching to cell surfaces (Buchanan and Pearce, 1979). Pili have been studied extensively in *P. aeruginosa*, primarily because of their involvement in pathogenesis (i.e., as components of virulence factors), although the structures of these cell appendages are not known for most other species. Pili have been observed also in strains of *P. alcaligenes*. On the other hand, no pili have been observed in cells of *P. chlororaphis*, *P. fluorescens*, *P. oleovorans* or *P. putida*.

The low G+C content of the pilin genes in *P. aeruginosa* suggests that they may have been transmitted by horizontal transfer and, as a consequence, have a different pattern of codon usage (West and Iglewski, 1988).

A common characteristic of the fluorescent pseudomonads is the production of pigments that fluoresce in short wavelength (254 nm) ultraviolet light, particularly after growth under iron limitation. Some of these pigments and their derivatives are known to act as siderophores in these bacterial iron uptake systems (Meyer et al., 2002). The production of pigments by *Pseudomonas* species is demonstrated by cultivating the bacteria in media such as King's medium B, which contains no added iron. The medium is also recommended for demonstrating the production of the nonfluorescent blue pigment, pyocyanin, characteristically produced by most strains of *P. aeruginosa*. While *P. aeruginosa* is the most notable species of *Pseudomonas* for the production of distinctive pigments, other spe-

cies are known to produce soluble and insoluble pigments that may be diagnostic. Pyoverdine (yellow-green fluorescence) represents the main siderophore type of *Pseudomonas* species. (Meyer and Hornsperger, 1998). The taxonomic value of siderophore-typing appears to lie in the species- and strain-specificities. The results of siderophore-typing of a large number of fluorescent and nonfluorescent strains correlated with the results of genotypic analyses, indicating that this approach is promising for differentiation at the species level (Fuchs, 2001; Meyer et al., 2002). Other pigments produced by species of *Pseudomonas* include pyocyanin (*P. aeruginosa*, blue color), pyorubin (*P. aeruginosa*, red color), oxochlororaphin (*P. aureofaciens*, orange color), chlororaphin (*P. chlororaphis*, green color), oxychlororaphin (*P. chlororaphis*, orange color), etc. (Hugh and Gilardi, 1980).

The basic morphological characteristics of *Pseudomonas* are common to many families of bacteria and so are of little value in the positive identification or diagnosis of members of the genus.

### Nucleotide Base Composition of Genomic DNA

The first genotypic analysis applied in bacterial characterization (Lee et al., 1956), the nucleotide base ratio of genomic DNA is measured as the ratio of the amount of guanine and cytosine nucleotides to the total amount of nucleotide bases (i.e., % G+C). Among prokaryotes, the G+C content ranges between 22–74 mol% (Marmur et al., 1963) and has proven to be useful for differentiating some taxa that are phenotypically similar. Although no exact guidelines have been established, generally, the G+C content has been reported to vary no more than 10 mol% within a bacterial genus (Stackebrandt and Liesack, 1993). Among the *Pseudomonas* species (*sensu stricto*), the range of G+C content is ca. 59–68 mol%, while among species reclassified within new genera, the range of G+C is 65–69 mol% for *Burkholderia* species, 64–68 mol% for *Ralstonia* species, 62–70 mol% for *Acidovorax* species, 62–67 mol% for *Comamonas* species, 65–67 mol% for *Brevundimonas* species, etc. (Palleroni, 1984). Though markedly different genomic DNA G+C contents certainly reveal different bacterial taxa, similar G+C contents do not necessarily indicate genomic relatedness.

### Ribosomal RNA Gene Sequences

During the last decade, a concerted movement in microbial taxonomy has adopted phylogenetic relationships inferred from genotypic analyses, particularly those derived from rRNA and

rRNA gene (rDNA) sequence analyses, as an important component of a taxonomic “framework”. Analyses of rRNA-rDNA sequence similarities of bacteria have been essential for elucidating their intergeneric relationships, and sequence determinations and analyses of 16S rRNA genes are among the recommended minimal standards for descriptions of new species. However, the application of rRNA-rDNA sequence analysis for determining intrageneric relationships of bacteria has not proven to be as successful. Given the early optimism for applying a principle of “one rRNA sequence type = one bacterial species,” the reality observed in the last decade has proven to be sobering in light of the conservative nature of the rRNAs, which have proven to be limited in resolving the fine bacterial phylogenetic structure below the genus level. Stackebrandt and Goebel (1994) assessed the relationships between genomic DNA-DNA similarity (determined by hybridization) and 16S rRNA gene sequence similarities, and they have described the levels of 16S rRNA gene sequence similarity (or dissimilarity) that indicates when a bacterium does not belong to a given species. However, a definition of bacterial species based upon 16S rRNA-rDNA sequence similarities still is not established.

The limitation in achieving definitive resolution of bacterial species does not suggest that 16S rRNA-rDNA cannot be useful for estimating intrageneric relationships. Suffice it to say that 16S rRNA gene sequences have been used to demonstrate considerable internal evolutionary structure within the spectrum of *Pseudomonas* species (sensu stricto; Moore et al., 1996; Anzai et al., 1997; Anzai et al., 2000). Moore et al. (1996) detected at least two distinct intrageneric divisions designated, for want of better labels, 1) the “*P. aeruginosa* intrageneric cluster” and 2) the “*P. fluorescens* intrageneric cluster.” Most species of *Pseudomonas* (isolated and characterized to date) group within one of these two clusters. Furthermore, *Pseudomonas* species were observed to be linked through distinct “evolutionary lineages” within the primary dichotomy of the genus. The same overall topography of the two major clusters, as well as the relationships of species comprising the evolutionary lineages, with some exceptions, has been observed in subsequent phylogenetic reconstructions (Anzai et al., 2000). Admittedly, the *Pseudomonas* intrageneric clusters and evolutionary lineages mentioned here comprise inferred natural branching orders. However, the organization of the two major intrageneric clusters, as well as the species comprising the evolutionary lineages, have been observed also in nonribosomal RNA-based analyses, using fatty acid methyl ester (FAME) and phospholipid fatty acid profiling

(Vancanneyt et al., 1996b), or sodium dodecylsulfate-polyacrylamide gel electrophoresis SDS-PAGE analysis of whole cell proteins (Vancanneyt et al., 1996a). Questions exist as to whether *P. putida* and closely related species, as well as some newly isolated species, are included as evolutionary lineages within one of the two primary intrageneric clusters or comprise separate, distinct intrageneric clusters.

*Pseudomonas pertucinogena* and “*P. denitrificans*” are seen to branch clearly outside the phylogenetic spectrum of all other species of the genus (Anzai et al., 2000), raising the question as to their taxonomic placement. “*P. denitrificans*” was placed on the list of nomina rejicienda (rejected synonyms) as a nomen ambiguum, because of previous confusion regarding its taxonomic position (Doudoroff et al., 1974b). Until comprehensive, polyphasic analyses of these two organisms has been carried out and their proper taxonomic positions confirmed, it is premature to include either species within the genus *Pseudomonas* (sensu stricto).

With the increase in *Pseudomonas* species, isolated and characterized by 16S rRNA gene sequence comparisons, the resolution between the individual sequences of the species has decreased and the branching orders have become less discernible. Such observations led to a significant problem in using rRNA-rDNA sequences for species identifications. Overall, 16S rRNA gene sequence similarities between species of *Pseudomonas* range from approximately 93% to 99.9% (given some unknown degree of error in sequencing results, 16S rRNA gene sequences of different species that may be identical cannot be ruled out). The complete 16S rRNA gene sequences are approximately 1540 nucleotides long. However, less than 10% (148 nucleotide positions) of the gene of *Pseudomonas* species appear to be susceptible to nucleotide base change and most of the positions that vary between species are located within hypervariable regions. Given these observations, if it were conceivable to apply the “one rRNA sequence type = one species” principle, the number of possible *Pseudomonas* species would be  $(4)^{148}$  (where 4 = the number of nucleotides that can exist at any given variable position, and 148 = the number of variable nucleotide positions). However, bacteria of different species have been observed to possess identical 16S rRNA gene sequences (Fox et al., 1992). With such an observation on record, one cannot assume that a unique 16S rRNA gene sequence will characterize a single bacterial species. This premise, of course, has important consequences for *Pseudomonas* taxonomy and the analyses of *Pseudomonas* in studies of microbial ecology and diversity.

Another potential genotypic target for resolving the species of the genus *Pseudomonas* are the 23S rRNA genes. In general, the limited number of sequences that have been determined have indicated that the larger (ca. 2500 nucleotides) bacterial 23S rRNA genes possess a higher degree of sequence variation than do the 16S rRNA genes (Olsen, 1988). Christensen et al. (1994) analyzed selected, variable target regions within the 23S rRNA genes of some *Pseudomonas* species, and the data failed to provide a reliable basis for identifying closely related species. However, the number of analyses on the 23S rRNA genes of bacteria, including species of the genus *Pseudomonas*, has remained limited, and, as a result, their value for resolving the fine intragenetic relationships is not yet established.

The similarities in rRNA gene sequences and the phylogenetic relationships may be used, in turn, for developing diagnostic tools for detecting and identifying bacteria at the genus and species levels in environmental or clinical samples. The high specificity of nucleic acid probes and the different regions of the rRNA molecule, containing variable numbers of conserved sites, provide ready targets for oligonucleotides labeled with an appropriate molecule for in vitro or in situ detection of bacteria at different taxonomic levels (Amann et al., 1995). Genus- and species-specific oligonucleotide probes have been designed (Schleifer et al., 1992; Amann et al., 1996) that enable the detection of combinations of pseudomonad species from the different proteobacterial classes. However, a single set of hybridization probes for differentiating all species of the various pseudomonad genera or all species of the genus *Pseudomonas* has yet to be successfully developed. While it may be theoretically possible to differentiate bacteria on the basis of a single base change in a probe target region, the sequence regions exhibiting the nucleotide differences (i.e., “signatures”) between species of a genus are limited in number, as well as in their structural availability to probing. Thus, Amann et al. (1995) have applied the practice of using nested probes in a “top-to-bottom” approach, whereby combinations of oligonucleotides used to differentiate organisms at different taxonomic levels systematically refine the levels of identification that may be determined. Such a strategy, which requires a large number of probes for identifying an organism of a specific sequence type, becomes realistic when used with micro-array hybridization systems.

In the same way that rRNA-rDNA sequence data have provided the means for developing hybridization probes as tools for differentiating bacterial species, the sequence data has also been used for devising PCR-diagnostic assays of high

specificity. Widmer et al. (1998) combined specifically targeted PCR-amplification assays with restriction fragment length polymorphism (RFLP) analyses to develop a rapid assay for detecting 16S rDNA sequence types that cluster within the phylogenetic spectrum of *Pseudomonas* species.

### Genomic DNA Similarities

Genomic DNA-DNA reassociation similarities have been accepted for many years as the molecular standard by which bacteria are classified at the species level (Johnson, 1984). The “cut-off” levels of DNA-DNA similarity that are used to “define” strains of a species range from above approximately 60% similarity ( $\delta T_{m(e)}$ , 6–9°C to approximately 70% similarity ( $\delta T_{m(e)}$ , 5°C; Brenner et al., 2001).

The original DNA-DNA hybridization experiments were performed by McCarthy and Bolton (1963) and were applied at Berkeley to the analysis of the pseudomonads to complement the existing phenotypic data. The results further supported previous conclusions based upon phenotypic analyses pointing to a significant degree of heterogeneity and fundamental differences among the species assigned to *Pseudomonas*, as the genus was known at that time. Subsequent analyses supported the subdivision of the genus (Johnson and Palleroni, 1989).

### rRNA Gene Intergenic Spacer (IS) Sequences

A potential target site for resolving intragenetic relationships of *Pseudomonas* may be found in the noncoding, intergenic spacer (IS) region between the 16S and 23S rRNA genes. This DNA is excised during the process of transcribing the ribosomal RNA during the synthesis of ribosomes and is, thus, exposed to less evolutionary conservation than the rRNA genes.

Tyler et al. (1995) were able to differentiate *P. aeruginosa* and *P. mendocina*, as well as different pseudomonad bacteria, to the species level by sequence comparisons of PCR-amplified 16S-23S rDNA IS regions. IS sequences from different strains of the same species did not exhibit sequence differences greater than 5% and, in most cases, were not more than 2% divergent. IS sequences of different species of the same genus exhibited significantly higher (25–20%) sequence dissimilarities.

Guasp et al. (2000) have used 16S-23S rDNA IS sequence characteristics to resolve the genovars of *P. stutzeri*, developing assays using restriction digestions of PCR-amplified IS regions, and correlating the results with differentiations determined by DNA-DNA hybridization

analyses. Although the IS sequence data for other species of *Pseudomonas* is sparse, these studies, and others analyzing various bacterial taxa, suggest that the results of sequence analyses of these DNA regions correlate well with results of DNA-DNA hybridization analyses and may offer an alternative to the DNA-DNA hybridization technique for identifying *Pseudomonas* and other bacterial species.

### Housekeeping Gene Sequences

The use of conserved protein-coding gene sequences as molecular chronometers has met with varied success. In general, the rates of change are significantly greater than those observed for rRNA genes and the sequences have been proposed as alternatives for estimating close (i.e., intragenetic) phylogenetic relationships (Santos and Ochman, 2004). Probably the most comprehensive studies employing housekeeping gene sequence targets in *Pseudomonas* have come from analyses of the *gyrB* gene that encodes the  $\beta$ -subunit protein of DNA gyrase (topoisomerase type II; Yamamoto and Harayami, 1998; Yamamoto et al., 2000). The base substitution rates of *gyrB* within species of the genus *Pseudomonas* were observed to be greater than those of the 16S rRNA genes of the same organisms, presumably because of the number of sites available for neutral base substitutions. Interestingly, while the branching order of the most closely related bacteria, such as different strains of *P. putida*, was clearly much more defined by *gyrB* gene sequence comparisons than could be achieved with 16S rDNA sequences (Yamamoto et al., 2000), the overall branching order of the range of different species of *Pseudomonas* was observed to be essentially the same.

Studies targeting other genes with purported evolutionary chronometer characteristics and applied to *Pseudomonas* include the *rpoD* gene for the  $\sigma^{70}$  factor of the RNA polymerase (Yamamoto and Harayama, 1998) and the *narG* and *nosZ* genes for nitrate reductase and nitrous oxide reductase, respectively (Delorme et al., 2003). However, the number of species and strains analyzed in these studies has been too limited to be able to determine whether these genes provide reliable reconstructions of *Pseudomonas* phylogeny.

Santos and Ochman (2004) have argued that problems associated with relying upon a single gene, such as the 16S rRNA gene, with its inherent limitations, to estimate organismal phylogenies may be circumvented by typing multiple single-copy gene loci of selected "chronometer" proteins. While such multilocus sequence typing

(MLST) analyses have been carried out on some bacterial taxa, this approach has yet to be applied to the analysis of *Pseudomonas*.

### Cell Lipid Compositions

The lipid composition of cell walls has been recognized to reflect the "natural relationships" between bacteria for nearly 40 years (Ikawa, 1967). Fatty acid, polar lipid, lipoquinone and polyamine profiles are regarded as valuable phenotypic markers of taxonomic value (Ratledge and Wilkinson, 1988; Tindall, 1994), although limited in their ability to resolve close relationships. Whole-cell fatty acid methyl ester (FAME) profile databases are now available as commercial products (e.g., the Microbial Identification System from Microbial ID, Inc. [Newark, DE]) and are used to identify bacteria at the genus level, in most cases, and subgenus levels in many cases.

In several laboratories, the compositions of cellular lipids of pseudomonads and other taxa have been described (Wilkinson et al., 1972; Moss and Dees, 1976; Ikemoto et al., 1978; Collins and Jones, 1981; Oyaizu and Komagata, 1983; Vancanneyt et al., 1996b). Whole-cell fatty acids of 16:0 and isomers of 16:1 and 18:1 appear to be typical of most Proteobacteria and are of limited diagnostic value in differentiating *Pseudomonas* (*sensu stricto*) from other genera (Vancanneyt et al., 1996b). *Pseudomonas* species have 3-OH 10:0, 3-OH 12:0 and 12:0, in addition to 16:1  $\omega$ 9c, 16:0 and 18:1 $\omega$ 7c, as their cellular fatty acids, as well as the Q-9 ubiquinone. Most species also have the saturated 12:0 2-OH component and no significant levels of 3-OH 14:0 (Vancanneyt et al., 1996b). A small number of species may also produce the 14:0 fatty acid. Although few analyses have been carried out on the polar lipid compositions of *Pseudomonas*, phosphatidyl glycerol, phosphatidyl ethanolamine, and diphosphatidyl glycerol (cardiolipin) have been observed as the major cell components, with a number of uncharacterized minor components also present. These comprise the typical cellular chemical compositions of *Pseudomonas* spp. (*sensu stricto*). The differential value of the lipid components of bacteria is found in the overall combinations of the lipid species, not necessarily in the presence of a single lipid biomarker. Furthermore, quantitative and qualitative differences in some lipid components (i.e., cellular fatty acids) also are valuable taxonomic markers for *Pseudomonas* species.

### Alginate Production

Alginate is an exopolysaccharide (EPS) comprised of a linear  $\beta$ -1,4-linked polymer of D-

mannuronic acid and L-gluronic acid. Alginate is produced by some bacteria, most notably by *P. aeruginosa* infecting the lungs of cystic fibrosis patients and causing severe respiratory problems (Govan, 1988), as well as by phytopathogenic or plant-associated fluorescent *Pseudomonas* species (Fett et al., 1986).

The genes involved in alginate synthesis in *P. aeruginosa* have been cloned and analyzed. Screening of bacteria for alginate genes, using DNA probes that target four genes involved in alginate production, detected DNA sequences homologous to all *P. aeruginosa* alginate genes in the chromosomal DNA of many species of *Pseudomonas* (*sensu stricto*), with the exception of *P. stutzeri*, which reacted to three of the four probes (Fialho et al., 1990). All of the alginate genes analyzed were detected in *Pseudomonas* species and the *Azotobacter-Azomonas* lineage, but fewer numbers of the genes were found in the other pseudomonad species and enteric genera.

In a related study, the *trans*-activation of gene *algU*, which controls the conversion of *P. aeruginosa* to a mucoid phenotype, was observed to be caused by a mutation in gene *algN*, at an adjacent locus, the product of which, presumably, inhibits the regulation of alginate production (Goldberg et al., 1993). *Pseudomonas* species were screened by hybridizing homologous DNA fragments to a probe for *algU* from *P. aeruginosa* (Dubnau et al., 1965). Homologous sequences were detected in the DNA of rRNA homology group I *Pseudomonas* species (*P. fluorescens*, *P. putida*, *P. mendocina* and *P. stutzeri*) but not in *Comamonas acidovorans* (rRNA group III), *Brevundimonas diminuta* (rRNA group IV), or *Stenotrophomonas maltophilia* (rRNA group V).

These data suggest that alginate production may be a useful indicator for differentiating *Pseudomonas* (*sensu stricto*) from other bacteria, including other phenotypically similar pseudomonads.

### Cellular Proteins

The comparison of amino acid sequences of selected homologous proteins offers the potential for assessing relationships among different bacterial genera (Schwartz and Dayhoff, 1978). However, protein sequence determinations are not a routine procedure. Thus, methods for determining the antigenic divergence of heterologous proteins, using antisera raised against reference proteins, can be used to identify amino acid sequence differences.

Glutamine synthetase, an important enzyme involved in nitrogen metabolism, has been the object of comparative immunological studies of

pseudomonads. The results of these studies were identical to those of the nucleic acid hybridization experiments (Baumann and Baumann, 1978). Other immunological studies of selected enzymes (such as muconolactone isomerase [Stanier et al., 1970], aliphatic amidases [Clarke, 1972], and histidine ammonia-lyases [Rokosu, 1983]) have determined the degrees of similarity among homologous proteins of pseudomonads. These studies have provided useful determinative applications and further confirmation of the internal subdivisions of *Pseudomonas* (Palleroni, 1975; Palleroni, 1993), although, in an immunological study of the relatedness of histidine ammonia-lyases (histidases) of *Pseudomonas* species, the anti-P antibody appeared to be diagnostic for fluorescent species of *Pseudomonas* but not for the nonfluorescent species (Robert-Gero et al., 1969).

Outer membrane proteins include three or four predominant proteins that can be discerned by electrophoresis and are conserved among species of rRNA homology group I *Pseudomonas* (Kragelund et al., 1996). An antibody raised against one of these proteins from a strain of *P. fluorescens* (OprF, 37 kDa) was used in Western blot screenings of isolates from rhizosphere samples, and specificity for rRNA homology group I *Pseudomonas* was confirmed.

Tesar et al. (1996) described another approach exploiting the immunological differentiation of characteristic cellular proteins in which whole cell protein electrophoresis patterns, reacted with antibodies of appropriate specificity in a Western blot, enable identifications over a wide phylogenetic range. Monoclonal antibodies generated against *Pseudomonas*-specific epitopes of outer membrane proteins were able to differentiate *Pseudomonas* species (*sensu stricto*) from species of closely related genera.

Such analyses appear to be applicable to different taxonomic levels, such as the differentiation of the genus *Pseudomonas* (*sensu stricto*), although the immunological approaches may be most useful for intrageneric identification, because of the potential for extremely high antibody specificity.

### Substrate Utilization

Substrate utilization has been an essential criterion for fine-resolution differentiation of species (and strains) of *Pseudomonas*. The lists of substrates utilized by *Pseudomonas* species are extensive, and the pathways for metabolism have been reviewed comprehensively (Stanier et al., 1966; Palleroni and Douderoff, 1972; Palleroni, 1984a; Palleroni, 1984b; Palleroni, 1993). Nutritional screenings of *Pseudomonas* species have been used to assess the capacity for growth at the

expense of a variety of carbon compounds (Stanier et al., 1966) and the findings have demonstrated the metabolic versatility that has made the genus renowned since the beginning of the twentieth century (den Dooren de Jong, 1926). In one case, a single strain of *P. putida* was observed to utilize 77 of 200 compounds tested, including carbohydrates, alcohols, saturated and unsaturated fatty acids, amino acids, amides and amines (den Dooren de Jong, 1926). In a separate study (Palleroni et al., 1973), another strain of *P. putida* grew on 80 of 150 compounds tested.

Many aromatic compounds are used by *Pseudomonas* species as growth substrates. During the early analyses at Berkeley, these compounds were observed to be typically metabolized to a common intermediate,  $\beta$ -keto adipate (3-oxoadipate), by the fluorescent species. Species associated with rRNA homology groups II-V used different pathways for the metabolism of various aromatic compounds, although the  $\beta$ -keto adipate pathway is also followed by pseudomonads and other bacteria unrelated to *Pseudomonas* species. (sensu stricto; Stanier, 1968). The mechanisms of aromatic ring cleavage appear to be characteristic for the different pseudomonad rRNA homology groups. By using antibodies against crystalline preparations of two enzymes of the  $\beta$ -keto adipate pathway, muconate lactonizing enzyme (muconate cyclisomerase) and muconolactone isomerase isolated from *P. putida*, heterologous reactions were observed in preparations from *P. putida* biovars, *P. aeruginosa*, all biovars of *P. fluorescens* and *P. stutzeri* but not in preparations from *B. cepacia* or species of the *Comamonas* group (Stanier et al., 1970).

## Metabolism of Amino Acids

Different pathways leading to L-phenylalanine or L-tyrosine are conserved in bacterial taxa (Byng et al., 1982) and analysis of key enzymatic features in these pathways, such as 2,4-diamino-6-hydroxypyrimidine (DAPH) synthase, prephenate dehydrogenase, arogenate dehydrogenase, prephenate dehydratase and arogenate dehydratase, have been used successfully to differentiate the members of pseudomonad rRNA homology groups (Byng et al., 1983). The multibranched pathway of aromatic amino acid synthesis offers not only a degree of variation in biochemical details not exhibited in the pathway of aliphatic amino acid synthesis, but also a richer source of regulatory information. For these reasons, Jensen and his collaborators exploited the interconnected pathways of aromatic amino acid synthesis as a model for the study of the phylogenetic relationships

among the groups of aerobic pseudomonads (Byng et al., 1980; Byng et al., 1982; Whitaker et al., 1981). The results of this work were in overall agreement with the subdivision of *Pseudomonas* into five distinct rRNA similarity groups.

Several pathways for the degradation of arginine by different species of the genus *Pseudomonas* are known. In one of the pathways, known as the arginine dihydrolase or arginine deiminase pathway, arginine is converted to citrulline and this, in turn, into ornithine and ATP, which allows the cells to maintain their motility for an extended time under anaerobiosis (Hills, 1940; Slade et al., 1954; Sherris et al., 1959). The presence of arginine deiminase is assayed by the disappearance of arginine or, more simply, an increase in the pH of the medium, owing to ammonia liberation (Thornley, 1960).

Pathways of arginine degradation in *Pseudomonas* are further characterized by key reactions: arginine dehydrogenase (oxidase) and arginine succinyl transferase in fluorescent species, as well as in species of other pseudomonad genera (Stalon and Mercenier, 1984; Stalon et al., 1987; Jann et al., 1988). The arginine dihydrolase system may no longer be considered to be characteristic of *Pseudomonas*, since it is detected also in species of other genera, and some *Pseudomonas* species (such as *P. stutzeri*) give a negative reaction. However, analysis of arginine dihydrolase, in combination with other tests, still can be of diagnostic value.

Arginine metabolism in *Pseudomonas* species with multiple catabolic pathways for its utilization as carbon and nitrogen sources is of particular importance for the control of metabolic integration in cells. Transcriptome analyses identified genes controlled by the arginine regulatory protein ArgR in *P. aeruginosa* (Lu et al., 2004). Ten putative transcriptional units of 28 genes were observed to be inducible by ArgR and arginine, indicating that ArgR is important in the control of arginine and glutamate metabolism and that arginine and ArgR may have redundant effects in the induction of uptake systems of certain compounds.

## Physiology

### Metabolism and Metabolic Pathways

The metabolism of *Pseudomonas* (sensu stricto) is traditionally recognized to be strictly respiratory, although the arginine deiminase pathway yields energy and is termed "arginine fermentation" and a form of pyruvate fermentation may be carried out by some species. All species respire aerobically, using oxygen as the terminal electron acceptor for oxidative phosphorylation.



Some species also have a supplementary anaerobic respiratory system, working concurrently with the aerobic pathway. The supplementary pathway, which has been a determinative characteristic for some species of *Pseudomonas*, uses nitrate ( $\text{NO}_3$ ) as the final electron acceptor (nitrate respiration). Denitrification via nitrate respiration is an energy-yielding catabolic process. Assimilatory denitrification of nitrate as a source of nitrogen for growth occurs through its reduction to ammonia.

The cytochromes present in the electron transport chains of some species of *Pseudomonas* have been characterized (Stanier et al., 1966), with absorption spectra detecting *a*-, *b*- and *c*-type cytochromes in most species, although some phytopathogenic species (e.g., *P. syringae*, *P. viridiflava*, *P. savastanoi*, etc.) lack the *c*-type cytochrome (Sands et al., 1967). These observations correlate with analyses of oxidase assays of *Pseudomonas* species, in which the *c*-type cytochrome is necessary for positive reactions (Jurtshuk and McQuitty, 1976).

The first step in the oxidation of many organic substrate compounds by *Pseudomonas* species is carried out by the action of oxygenases, incorporating molecular oxygen into the chemical structure of the compound. Oxygen is a requirement for these reactions. The oxygenases form a diverse class of enzymes (mono- and dioxygenases), differing in structure, specificity and mechanism, and catalyze the oxidation of an extensive number of compounds, including hydrocarbon contaminants. Within the University of Minnesota Biocatalysis-Biodegradation Database (<http://umbbd.ahc.umn.edu>), more than 30% of the total number of enzymes catalogued are oxygenases, many derived from *Pseudomonas* species (Wackett, 2003).

As mentioned previously, some species of *Pseudomonas* are able to use nitrate as an alternative electron acceptor. Nitrate respiration is dependent upon the activities of nitrate and nitrite reductases, which are induced in the presence of nitrate under anaerobic conditions and may be repressed by oxygen. Thus, levels of oxygen may play an important role in determining the success of denitrifying populations of *Pseudomonas* species in various habitats, for example, in well-aerated soils.

Additionally, a new species of *Pseudomonas* was described recently, *P. chloritidismutans*, which is able to utilize chlorate ( $\text{ClO}_3^-$ ) as an alternative energy-yielding electron acceptor, in addition to oxygen (Wolterink et al., 2002). The ability to consume and oxidize nitric oxide (NO) to nitrate under oxic conditions has been observed in one *Pseudomonas* species. The mechanism of oxidation remains unclear, although it appears to be associated to detoxifi-

cation or co-oxidation rather than an energy-yielding process. Although *Pseudomonas* has been studied for more than a century, new catabolic pathways are being detected in new isolates. The tricarboxylic acid (TCA) cycle in *Pseudomonas* is central in the regulation of cell catabolism and biosynthesis in all species. Species of *Pseudomonas* tend to utilize organic acids in preference to more complex organic compounds and this, in turn, represses many inducible peripheral catabolic enzymes. All or most of *Pseudomonas* species have incomplete glycolytic pathways, lacking 6-phosphofructokinase. *Pseudomonas* species dissimilate sugars and organic acids preferentially through the Entner-Doudoroff pathway. Hexoses and related compounds are converted to glyceraldehyde-3-phosphate and pyruvate via the Entner-Doudoroff pathway and various peripheral pathways, in which 6-phosphogluconate is a key intermediate (Eisenberg et al., 1974). All genes for the pentose phosphate pathway, the TCA cycle, the glyoxylate shunt, as well those for the oxidative and electron transport chain, are present in the genomes of the species of *Pseudomonas* whose genomes have been sequenced.

*Pseudomonas* species are able to use amino acids as carbon and nitrogen sources. When amino acids are present, the cell activates a specific membrane permease, which provides the transport mechanism for the amino acids to cross into the cytoplasmic space. Using amino acids as nutrient sources saves the cell energy, as the amino acids are immediately usable, requiring little or no modification to be incorporated directly into the synthesis of cell biomass.

Some defining physiological characteristics have not been critically tested in all members of the genus, and as a consequence, occasional reports of exceptional strains have been noted. Thus, nitrogen fixation has been reported to occur in *P. stutzeri*, and *P. aeruginosa* may be capable of growing anaerobically, albeit slowly, with arginine and small amounts of yeast extract.

## Genetics

Almost 10% of the genes in *P. aeruginosa*, *P. putida*, *P. syringae* and *P. fluorescens* genomes encode products involved in gene regulation, which reflects the evolutionary emphasis of this genus in monitoring and responding to a large number of environmental signals. The major regulatory control in bacteria of this genus is at the level of transcription, although an increasing number of posttranscriptional control systems are also becoming evident in this genus, to fine-tune the levels of expression of certain proteins.

This section summarizes some of the features involved in the transcriptional machinery and general regulatory circuits in *Pseudomonas*.

**SIGMA FACTORS IN *PSEUDOMONAS*** In *Pseudomonas*, core RNA polymerase can choose among a large number of sigma factors to transcribe the extensive variety and number of genes that species of this genus possess. *Pseudomonas* has a major sigma factor,  $\sigma^{70}$ , that recognizes promoters controlling the expression of housekeeping genes. Domínguez-Cuevas and Marqués (2004) compiled 149  $\sigma^{70}$ -dependent *Pseudomonas* promoters, in which the transcriptional start point had been determined experimentally. They found that these promoter regions are significantly richer in AT pair content (approximately 50%) than the genomes from which they are derived (33.4% to 38.4%). The pattern resembles the consensus described for *Escherichia coli*, i.e., two conserved hexamers centered at positions -10 and -35 relative to the transcription start site. The -10 element in *P. aeruginosa* is TAtAAT and slightly different from that in *P. putida* (TATAcT), whereas the -35 element in both species is TTGACC. The spacer sequence between the -10 and -35 elements has an average length of 17 bp, although a 1-bp deviation of this length occurs with some frequency.

Multiple open reading frames (ORFs) coding for putative extracytoplasmic function (ECF) sigma factors that constitute a phylogenetically and functionally distinct subgroup within the  $\sigma^{70}$  family have been found in the genomes of *Pseudomonas* (Martínez-Bueno et al., 2002). Among these sigma factors are RpoS (sigma-38), RpoH (sigma-32), FliA (sigma-27), AlgU (sigma-22, homologous to RpoE in *E. coli*), PvdS and others. They control several iron uptake pathways, alginate biosynthesis, expression of virulence factors, tolerance to several stresses, expression of outer-membrane porins, etc. (Venturi et al., 1995; Brinkman et al., 1999; Burger et al., 2000; Rowen and Deretic, 2000; Schnider-Keel et al., 2001; Martínez-Bueno et al., 2002; Visca et al., 2002; Beare et al., 2003). Two ECF systems have been well characterized in *P. aeruginosa*: 1) AlgU, involved in the regulation of alginate biosynthesis, conferring a mucoid phenotype (Govan and Deretic, 1996), and directing the expression of a gene encoding the major heat shock sigma factor  $\sigma$  (thus, it likely plays a role in global gene regulation; Schurr and Deretic, 1997) and 2)  $\sigma^{\text{PvdS}}$ , a sigma factor involved in the regulation of pyoverdine siderophore biosynthesis.

The  $\sigma^{\text{E}}$  ECF factor of *P. aeruginosa* is encoded by the *algU* gene, which is part of the *algU-mucA-mucB-mucC* gene cluster.  $\sigma^{\text{E}}$  activity is controlled by the membrane-localized MucA

protein, which appears to be stabilized by the periplasmic protein MucB, although the precise function of MucC is still unknown (Boucher et al., 2000). In addition to the alginate biosynthesis genes, transcriptional microarray profiles of  $\sigma^{\text{E}}$ -dependent expression in *P. aeruginosa* revealed that the following genes are part of the  $\sigma^{\text{E}}$  circuit, namely: *pfpI*, encoding a putative protease; *osmE*, encoding an osmotically inducible lipoprotein; genes encoding several membrane proteins; genes encoding metabolic proteins; and genes encoding proteins involved in adhesion and in drug resistance (e.g., efflux pumps).  $\sigma^{\text{E}}$  has also been reported in a plant growth-promoting *P. fluorescens* strain and shown to be important for tolerance to osmotic and desiccation stress, although, unlike *P. aeruginosa*, it did not play a role in protection against heat damage (Schnider-Keel et al., 2001). The  $\sigma^{\text{E}}$ -regulated promoters exhibit the following consensus sequence [(-35) GAACTT—N16/17—(-10) TctgA].

Several ECF sigma factors are involved in iron uptake, a function that appears to be crucial for the ecological fitness of all *Pseudomonas* (Martínez-Bueno et al., 2002; Venturi et al., 2004). The best characterized of these iron uptake sigma factors is PvdS, which initiates transcription of the genes required for the biosynthesis of pyoverdine (Lamont et al., 2002; Beare et al., 2003). The signaling pathway leading to  $\sigma^{\text{PvdS}}$  activation involves the ferri-pyoverdine outer membrane receptor, FpvA, which, upon interaction with ferric-pyoverdine, transmits a signal to the anti- $\sigma^{\text{PvdS}}$  factor, FpvR, releasing the suppression of  $\sigma^{\text{PvdS}}$  and resulting in initiation of the transcription of pyoverdine genes and the *toxA* and *prpL* genes in *P. aeruginosa* (Lamont et al., 2002). A three-component system similar to FpvA/FpvR/ $\sigma^{\text{PvdS}}$  has been found in *P. putida* KT2440. Orthologues of  $\sigma^{\text{PvdS}}$  have also been identified to regulate siderophore production in *P. fluorescens* (Sexton et al., 1995). The *P. aeruginosa* PvdS protein has been shown to bind to several *pvd* promoters in which a DNA sequence at the -35 region, designated the "IS box" (G/C G/C TAAAT T/A C/G), is important for proper promoter functioning through specific recognition by this sigma factor (Wilson et al., 2001).

All *Pseudomonas* genomes have an *rpoN* gene encoding the  $\sigma^{54}$  factor that constitutes a group of its own, with characteristics that differ greatly from the  $\sigma^{70}$  family (Valls et al., 2004). As in other Gram-negative bacteria, promoters of this kind typically span DNA segments of approximately 150–200 bp. The most distinctive feature of the  $\sigma^{54}$ -systems is the presence of sequences that are characteristic for binding of the  $\sigma^{54}$ -containing RNAP, the so-called "-12/-24 motifs." These

include GG and GC doublets at positions -24 and -12, respectively, instead of the typical -10 and -35 hexamers of the  $\sigma^{70}$ -promoters. The second major functional motif in  $\sigma^{54}$ -promoters is the binding site(s) for the cognate activators. These sites may be located at various distances (50–150 bp) upstream from the -12/-24 motif. In addition to the -12/-24 and the upstream activating sequence (UAS), many (though not all) promoters have an integration host factor (IHF)-binding site at the intervening region. IHF binding to DNA sharply bends the target sequence, an event that has multiple consequences. Activation of this type of promoter is mediated mainly by members of the NtrC-family of regulators (see below). A feature to highlight is that the number and functions of the gene products of the genes transcribed by  $\sigma^{54}$  vary tremendously among the different *Pseudomonas* strains. However, a common characteristic is that the *rpoN* mutants of *P. aeruginosa* (Totten et al., 1990) and *P. putida* (Kohler et al., 1989) lack glutamine synthetase and fail to produce urease. *P. syringae* and *P. aeruginosa rpoN* mutants are less virulent than the corresponding wildtype strains. In the case of the plant pathogen, this is because it fails to synthesize the phytotoxin coronatine, and in *P. aeruginosa*, it is because the RpoN mutant fails to synthesize a number of virulence factors (Hendrickson et al., 2001). Last but not least, *rpoN* is required for the production and assembly of the flagellum.

**FAMILIES OF REGULATORS** The LysR transcriptional regulator family is the largest paralogous group in *Pseudomonas* genomes, comprising more than 100 members, which is a remarkably high value for prokaryotes (normally ranging between 2 and 12) and comparable only to the situations in *Sinorhizobium meliloti* (86), *Agrobacterium tumefaciens* (75), and *Escherichia coli* strains (45–60). LysR-type regulators are associated with the regulation of many diverse functions, and play a central role in the activation of the expression of enzymes and proteins involved in aromatic metabolism, such as in protocatechuate (PcaQ) and catechol catabolism (CatR), and of other soil- and plant-related functions. AraC transcriptional regulators control processes such as carbon metabolism, stress response, and pathogenesis and, with more than 30 members, are also highly represented in the genomes of *Pseudomonas*. Members of the NtrC family that work with  $\sigma^{54}$  are also abundant in all *Pseudomonas* (around 25 members) and work in many different regulatory circuits (Valls et al., 2004). In *P. aeruginosa*, these regulatory circuits include the PhhR regulator for phenylalanine metabolism (Song and Jensen, 1996), and the

regulator of the *lipA* gene, encoding a powerful lipase (Jaeger et al., 1996). In different species of the genus *Pseudomonas*, a number of catabolic pathways for the metabolism of aromatic compounds, i.e., phenol, toluene/*m*-xylene, *o*-xylene and 2-hydroxybiphenyl, are under the control of  $\sigma^{54}$  regulators such as DmpR, XylR, TouR and HbpR (Ramos et al., 1997; Arengi et al., 2001; Jaspers et al., 2001; Shingler, 2004; Valls et al., 2004).

The genomes of *Pseudomonas* often contain a diversity of other regulatory gene families, such as repressors of the TetR and IclR family, which often regulate the expression of resistance to antibiotics, detergents and solvents. In all *Pseudomonas* genomes, members of the following families have been found: *asnC*, *gntR*, *lacI*, *luxR*, *Cro/cI*, *merR*, *marR* and *fis*.

**TWO-COMPONENT PHOSPHORELAY SYSTEMS INVOLVED IN SENSING NUTRIENTS AND STARVATIONS** All strains of *Pseudomonas* species contain a large number of two-component phosphorelay systems (TCSs). These systems consist of a histidine-kinase sensor and a response regulator (RR). Variations on the histidine-kinase (HK) sensor system have been reviewed recently by Filloux et al. (2004). In short, the sensor autophosphorylates in response to environmental stimuli and then transduces the signal to a response regulator, belonging to one of the above families, which is, in turn, activated upon phosphorylation. Examples of TCSs include the FleS/FleR pair that regulates motility and adhesion to mucins in *P. aeruginosa* (Richtings et al., 1995) and the PilS and its RR PilR that regulate transcription of the pilin gene *pilA* (Hobbs et al., 1993). CrbA is a classical HK which functions in unison with CrbB (Nishijyo et al., 2001) to control several specific metabolic pathways, and modulates the catabolism of various natural substrates in response to different carbon-nitrogen (C/N) ratios. Nitrogen nutrient deprivation also involves the TCS system NtrB–NtrC, while PhoR–PhoB is involved in phosphate assimilation. As proof of the complexity of iron metabolism in *Pseudomonas*, two TCSs have been shown to play an important role in iron acquisition in *P. aeruginosa*. The PfeS–PfeR pair is required for the enterobactin-inducible production of the ferric enterobactin receptor PfeA (Dean and Poole, 1993), and the PirR–PirS system is used as a second low-affinity ferri-enterobactin uptake system (Vasil and Ochsner, 1999). The GacS sensor is involved in the regulation of virulence in *P. aeruginosa* and works with the RR GacA (Reimann et al., 1997). Some relevant issues of this system are described below.

**CATABOLITE REPRESSION IN PSEUDOMONAS**  
When strains of *Pseudomonas* species are exposed to a mixture of potential carbon sources, they often assimilate them in an orderly fashion. In fact, most *Pseudomonas* species metabolize many organic acids or amino acids in preference to sugars, i.e., when *Pseudomonas* is confronted with succinate and glucose, the enzymes for glucose metabolism are not induced until succinate is exhausted (Collier et al., 1996). Nevertheless, glucose is known to repress the metabolism of mannitol and histidine. In contrast to other bacteria, catabolite repression is not mediated by cAMP but rather by the integration of several signals (Rojo and Dinamarca, 2004). Although the molecular basis for catabolite repression is, as yet, not well understood, evidence gathered in different laboratories points towards a series of proteins involved in signal integration. For instance, the Crc protein is involved in the repression of the genes involved in metabolism of sugar, amino acid, and nitrogenated compounds in *P. aeruginosa* and *P. putida*. Crc controls the metabolism of branched-chained amino acids and alkanes (MacGregor et al., 1996; Canosa et al., 2000; Yuste and Rojo, 2001). This protein is also said to be involved in biofilm development in *Pseudomonas* (O'Toole et al., 2000). A surprising finding is that catabolite repression of phenol metabolism (Petruschka et al., 2001) in *P. putida* strain H, and alkane metabolism in *P. putida* strain GPo1 is alleviated by the inactivation of the cytochrome *o* ubiquinol oxidase (encoded by *cyoABCDEF*). Cells are believed to sense the redox state of the respiratory chains, triggering catabolite repression processes.

Sigma-54-regulated Pu and Po promoters for the metabolism of toluene and xylenes and phenol encoded by plasmid pWW0 and pVII150 (Powlowski and Shingler, 1994; Ramos et al., 1997) are also under catabolite repression. Indeed, these promoters are not expressed during the exponential growth phase in rich media in the presence of pathway substrates (Marqués et al., 1994; Cases et al., 1996; Sze et al., 1996). Both promoters respond to the alarmone (p)ppGpp, although Po is more sensitive than Pu (Sze and Shingler, 1999; Carmona et al., 2000). The level of the alarmone is low in the exponential phase and increases in the stationary phase. The Pu promoter is also down-modulated in response to glucose in a process that involves the activities of the PtsN and PtsO proteins (Cases et al., 1999; Cases et al., 2001). Inactivation of *ptsN* makes Pu unresponsive to repression by glucose, although glucose metabolism is not affected in the mutant. On the contrary, inactivation of *ptsO* inhibits Pu activity regardless of the presence of glucose (Cases et al., 2001). The

Po promoter is not significantly influenced by PtsN-mediated glucose repression (Sze et al., 1996; Sze et al., 2002).

**QUORUM SENSING** In many strains of *Pseudomonas* species, production of secondary metabolites, rhamnolipids, extracellular enzymes and virulence factors is controlled by a cell-cell signaling system that is generally described as "quorum sensing" (QS) or "density-dependent gene regulation" (Fuqua et al., 1994; Fuqua et al., 1996; Salmond et al., 1995; Sitnikov et al., 1995). The basis of this system is that the bacterium produces autoinducer or signal molecules such as *N*-acyl homoserine lactones (HSLs) that accumulate in the growth medium and trigger the expression of target genes when a threshold concentration is reached.

In *P. aeruginosa*, the QS circuit is composed of the *lasRI* and the *rhlRI* systems (Van Delden and Iglewski, 1998). The *lasRI* system is composed of *lasI*, the autoinducer synthase gene responsible for the synthesis of 3-oxo-C<sub>12</sub>-HSL (*N*-[3-oxododecanoyl]-L-homoserine lactone), and *lasR* which encodes the transcriptional regulator LasR (Gambello and Iglewski, 1991; Passador et al., 1993; Pearson et al., 1994). The *rhlRI* system is composed of *rhlI*, the C<sub>4</sub>-HSL (*N*-butyrylhomoserine lactone) autoinducer synthase gene, and *rhlR*, which encode the transcriptional activator RhlR (Ochsner et al., 1994a; Ochsner et al., 1994b; Latifi et al., 1995; Pearson et al., 1995; Winson et al., 1995). At low cell density, the autoinducers 3-oxo-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL are synthesized at basal levels and diffuse, or are transported into the surrounding media where they become diluted; as a consequence, no gene transcription occurs (Pearson et al., 1999). With increasing cell density, the two autoinducers accumulate until their intracellular concentration reaches a threshold level. At this critical concentration, they bind to their corresponding regulatory protein (Fuqua et al., 1996). The regulator protein-autoinducer complex binds to specific DNA sequences upstream of the target genes enhancing their transcription (Stevens et al., 1994; Stevens and Greenberg, 1997). These systems, therefore, allow bacteria to communicate with each other (cell-to-cell signaling), to sense their own density (quorum sensing), and to behave in a coordinated manner, expressing specific genes as a population rather than as individual cells (Van Delden and Iglewski, 1998). The *lasRI* system regulates *lasB* expression and is required for optimal production of other extracellular virulence factors such as LasA protease and exotoxin A (Gambello et al., 1993). This system has also been shown to induce the transcription of the *xcpP* and *xcpR* genes that encode

proteins of the *P. aeruginosa* secretory pathway (Chapon et al., 1997).

The *rhlRI* system regulates the expression of the *rhlAB* operon that encodes a rhamnosyltransferase required for rhamnolipid production (Ochsner et al., 1994a), and is involved in the optimal production of LasB elastase, LasA protease, pyocyanin, cyanide, and alkaline protease (Brint and Ohman, 1995; Latifi et al., 1995; Pearson et al., 1997; Reimmann et al., 1997).

Both QS systems are highly specific in the sense that the respective autoinducers are unable to activate the transcriptional activator protein of the other system (Latifi et al., 1995; Pearson et al., 1997). However, both quorum-sensing systems are not completely independent of each other. The LasR/3-oxo-C<sub>12</sub>-HSL complex activates the expression of *rhlR* placing the *lasRI* system in a QS hierarchy above the *rhlRI* system (Latifi et al., 1996; Pesci et al., 1997).

Transcriptome analysis has shown that QS controls not only virulence in *P. aeruginosa* but also many other genes involved in basic cellular processes, such as DNA replication, RNA transcription and translation, cell division, and amino acid biosynthesis. Another set of genes under QS control are those of chemotaxis and biofilm formation (Davies et al., 1998; De Kievit et al., 2001). Rhamnolipids, which depend on the *rhlRI* system, are required for the maintenance of biofilm architecture (Davey et al., 2003). The timing of QS activation is a continuum. Some genes are activated during early growth phase, whereas others are activated during exponential growth, or even during the stationary phase. This timing is not dependent on the concentration of autoinducer but on the availability of the transcriptional regulator proteins (LasR and RhlR; Schuster et al., 2003; Vasil, 2003).

The regulation of the QS circuit itself is complex and involves several other transcriptional regulators. The *lasRI* system is regulated both positively and negatively at several levels. It has been shown to be positively controlled by the global response regulator GacA (which together with the sensor kinase GacS forms a two-component system; Reimmann et al., 1997), as well as by Vfr (a homologue of the global transcriptional regulator CRP), which is required for the transcription of *lasRI*. The RsaL inhibitor represses the transcription of *lasI*. QscR has been shown to inhibit the premature activation of *lasI* expression at low cell density (Chugani et al., 2001). Expression of the QS system is influenced by the RpoS and RpoN sigma factors, the global posttranscriptional regulator, RsmA, and the alarmone (ppGpp; Latifi et al., 1996; Whiteley et al., 2000; Pessi et al., 2001; Van Delden et al., 2001). Adding to this complexity is the finding of a *Pseudomonas* quinolone signal

(PQS) that depends on the QS circuit for its production, and which positively regulates *rhlI*, *rhlR* and *lasR* expression and induces *lasB* transcription (Pesci et al., 1999; McKnight et al., 2000; Calfee et al., 2001; Gallagher et al., 2002).

Phenazine biosynthesis is regulated by QS in *P. aureofaciens*, *P. chlororaphis* and *P. aeruginosa* (Pierson et al., 1994; Brint and Ohman, 1995; Wood and Pierson, 1996; Chin-A-Woeng et al., 2001). Expression of the phenazine biosynthetic operon in *P. aureofaciens* is controlled by PhzI and PhzR, which are members of the family of QS regulators (Wood and Pierson, 1996). Target regulatory boxes are located upstream of both the *phzI* gene and the *phz* biosynthetic operon, enabling activation of transcription in response to high concentration of the autoinducer C<sub>6</sub>-HSL. The central importance of QS for this strain is shown by the data that phenazine production is greatly reduced in a *phzI* mutant and is restored by addition of C<sub>6</sub>-HSL (Wood et al., 1997). GacS/GacA system regulates phenazine production (Haas et al., 2000) via transcriptional regulation of the *phzI* gene (Chancey et al., 1999). The level of *phzI* expression in a *gacA* or a *gacS* mutant is very low.

**POSTTRANSCRIPTIONAL CONTROL IN *P. FLUORESCENS*** In the plant-beneficial strain *P. fluorescens* CHA0, the *hcnABC* operon, which encodes hydrogen cyanide synthase, is regulated at the posttranscriptional level, and involves the translational regulatory proteins RsmA and RsmE (Blumer et al., 1999; Haas et al., 2002; Heeb et al., 2002; Haas and Keel, 2003). Evidence for this mechanism comes from measurements of the expression of transcriptional and translational studies in different genetic backgrounds. The current model proposes that GacS/GacA activates expression of target genes at the posttranscriptional level by inducing the transcription of at least two small, untranslated regulatory RNAs, termed "RsmY" and "RsmZ," which alleviate the repressive effects of RsmA (Heeb et al., 2002; Valverde et al., 2003) and of RsmE (Pessi and Haas, 2004). RsmA and RsmE seem to bind to the mRNAs of target genes blocking their translation (Blumer et al., 1999). Mutational analysis of the *hcnA* untranslated mRNA leader region suggests that about ten nucleotides surrounding the Shine-Dalgarno sequence (GGA) are the target recognized by RsmA.

### Genomics

As of June 2004, the genomes of four species of *Pseudomonas* have been sequenced and analyzed (Stover et al., 2000; Nelson et al.,

2002; Buell et al., 2003). At the time of this writing, the sequence for *P. fluorescens* was not published (see [http://www.sanger.ac.uk/Projects/P\\_fluorescens/](http://www.sanger.ac.uk/Projects/P_fluorescens/){The Wellcome Trust: Sanger Institute Web site}). The four species (*P. aeruginosa* strain PAO1, *P. putida* strain KT2440, *P. syringae* pv. tomato strain DC3000, and *P. fluorescens* strain SBW25) are derived from distinct phylogenetic lineages within the genus and include, respectively, an important opportunistic human pathogen, a strain used as a model system for biodegradation and other biotechnological applications, an important agricultural phytopathogen, and a plant growth-promoting strain colonizing roots and leaves.

The genomes of *Pseudomonas* species have approximately 6 Mbp (*P. aeruginosa*, 6,264,403 bp; *P. putida*, 6,181,863 bp; *P. syringae*, 6,397,126 bp; and *P. fluorescens*, approximately 6,700,000 bp), and approximately 5500 putative genes have been detected. These genomes are among the largest of the bacterial genomes that have been sequenced to date and the G+C contents of these genomes are among the highest: *P. aeruginosa*, 66.6%; *P. putida*, 61.5%; *P. syringae*, 58.4%; and *P. fluorescens*, 60.0%. Interestingly, the G+C contents determined by sequencing match almost exactly the G+C contents estimated previously for these species (Palleroni, 1984).

Detailed comparisons of the genomes of the four species of *Pseudomonas* have been compiled by Jensen et al. (2004). As this chapter is devoted to nonpathogenic aspects of *Pseudomonas*, the focus will be centered on data derived from the *P. putida* genome-sequencing project (Martins dos Santos et al., 2004; also see {TIGR Comprehensive Microbial Resource}).

*Pseudomonas putida* is one of the best studied species of the genus, although information on genetic organization, gene transfer, metabolism and regulation, catabolic abilities, etc., is fragmented and dispersed, providing a restricted and incomplete view of the metabolic properties and capabilities that determine its lifestyle and niche specificity. The biology of *Pseudomonas* species, their metabolic versatility, behavior under varied environmental conditions, genetic variability, and interactions with other organisms and the environment can be assessed and appreciated only within the biological context in the diverse ecological niches where they reside. *P. putida* strain KT2440 is a paradigm of the metabolically versatile species and is used worldwide as a model system for genetic and physiological studies, and for the development of biotechnological applications. *P. putida* KT2440 has been certified by the Recombinant DNA Advisory Committee of the United States National Institutes of Health as the host strain of a host-vector bio-

safety (HV1) system for gene cloning (Federal Register, 1982).

#### GENOME FEATURES AND GENETIC PLASTICITY

The 6.2-Mb genome of *P. putida* KT2440 contains 894 paralogous gene families, which is considerably higher than that of the 6.3-Mb genome of *P. aeruginosa* strain PAO1, which has 809 putative paralogs. This large number of paralogous families, some 50% more than so far found in other large bacterial genomes is indicative of the high degree of functional versatility of *P. putida*. The most abundant of the putative genes include the LysR transcriptional regulator family with 110 members, the response regulator receiver domain with 94 members, and the ABC transporter family with 93 members. Interestingly, the 27 families represented by more than 20 members are related to proteins involved in transcription regulation (LysR, AraC, TetR, Sigma-54, etc.), transport (ABC, major facilitator family [MFS], binding-protein-dependent transport system, and porins), environmental signaling (response regulator receiver domains, histidine kinases, PAS domain S-boxes, TonB receptors, among others) or catabolism (dehydrogenases, hydrolases, transferases, oxygenases, etc.). These features reflect the emphasis in *P. putida* on powerful cellular mechanisms that enable it to thrive in diverse environments and to compete successfully with other organisms. Of the remaining putative gene families, 770 of them (86%) have 7 or fewer members, and 537 (60%) have only 3 (171) or 2 (513) members, which is similar to other large bacterial genomes. This suggests that selection for environmental versatility has favored expansion of genetic capability through the development of numerous small paralogous families, whose members encode distinct functions, rather than by increasing the number of family members (Stover et al., 2000).

The *P. putida* KT2440 genome contains other features that reflect high functional diversity. For instance, 508 ORFs were identified as putative duplications that arose after *P. putida* diverged from a common evolutionary lineage with *P. aeruginosa*, with which it shares approximately 85% of its genome (Nelson et al., 2002). As in other studies, comparison of the *P. putida* and *P. aeruginosa* genomes suggests that most lineage-specific genes are expansions of paralogous gene families. Of the duplications found in *P. putida* KT2440, 111 (21.8%) are conserved hypothetical proteins, and 110 (21.6%) are hypothetical proteins that probably relate to the unique biology of this organism. A large family of these duplications includes 22 transposase genes resembling members of the IS4 and IS110 gene families (see

below). Lineage-specific gene duplications may reveal species-specific adaptations to habitat, since they are often accompanied by functional diversification and divergence (Tettelin et al., 2001). Indeed, of the 42 transposase and maturase genes that are present in KT2440, 24 (57%) are flanked by genes involved in energy metabolism, implying an association between these mobile elements and the metabolic capabilities of *P. putida* KT2440. This association may serve to mediate enhanced horizontal transfer or altered expression of the corresponding metabolic genes, or both.

Insertion sequence (*IS*) elements are frequently associated with genes and operons encoding accessory (i.e., non-housekeeping) functions, such as catabolic enzymes and pathways (Mae and Heinaru, 1994; Wyndham et al., 1994; Tan, 1999), pathogenicity factors (Weinel et al., 2002), and protective functions against noxious agents, such as antibiotics, biocides, detergents, etc. (Wery et al., 2001). Moreover, *IS* elements mediate gene rearrangements, and facilitate the dissemination by horizontal transfer of such gene clusters among bacterial populations (Mahillon and Chandler, 1998). The *P. putida* KT2440 genome contains a large number of *IS* elements belonging to several families, although, of the previously described transposable elements from plasmids of *Pseudomonas* species, only Tn4652, which was previously shown to be responsible for inserting plasmid genes into the *P. putida* chromosome (Mae and Heinaru, 1994), could be identified within the genome sequence. The abundance and diversity of *IS* and other transposable elements in the *P. putida* KT2440 genome underscores the importance of the accessory functions of this organism and is indicative of its genetic plasticity.

Many other atypical regions, most containing mobile elements and determinants of catabolic pathways, have been identified in the *P. putida* KT2440 genome. Codon composition analysis revealed 39 regions of the genome greater than 10 kb in length that have atypical composition, the majority of which also have a G+C content that is significantly different from that of the rest of the genome (Nelson et al., 2002). Many of these regions encode bacteriophage genes, transposable elements, or determinants for proteins involved in the metabolism of aromatic compounds. In total, 90 transposon and 92 prophage determinants have been detected.

Inspection of the *P. putida* KT2440 genome reveals that a 35-bp sequence, having the structure of an imperfect palindrome, is repeated more than 800 times throughout the genome, which has not previously been reported for

prokaryotes other than those of the Enterobacteriaceae family (Aranda-Olmedo et al., 2002). More than 80% of these sequences are extragenic and have thus been designated "Repetitive Extragenic Palindromic" ("REP") sequences. Of these, 82% are situated within 100 bp (and many within 30 bp) of the end of a neighboring gene. Given the positions and distribution of REP sequences in the *P. putida* genome, Aranda-Olmedo et al. (2002) suggested that they may serve to allow DNA gyrase to bind and relax DNA when excessive supercoiling is generated, thereby playing an important role in the regulation of gene expression.

**HABITAT-SPECIFIC FUNCTIONS** The genome of *P. putida* KT2440 is a complex mosaic structure with numerous and varied mobile elements, exhibiting features characteristic of terrestrial, rhizosphere and aquatic bacteria and suggests that acquisition of the combination of such features has equipped *P. putida* with its ability to thrive in diverse, often inhospitable environments, either free-living or in close association with plants.

**DILUTE ENVIRONMENTS.** An important characteristic of bacteria adapted to nutrient-poor aquatic lifestyles is the possession of high affinity transporters for substrates and nutrients. In addition to the relatively large number of high affinity transporters (most with an unknown substrate specificity), *P. putida* KT2440 has at least two ferric siderophore-transport systems and three ABC iron transporters, as well as 18 outer membrane ferric and ferric-related siderophore-receptors, three phosphate and two sulfonate ABC transport systems (in addition to a periplasmic sulfonate protein), as well as various transporters for sulfate, nitrate, common oligo-elements, and metals. The existence of multiple transport systems for iron and phosphate is consistent with the need to overcome phosphate and iron limitation in dilute environments and the capacity of *P. putida* to deal with the diversity of micronutrients in heterogeneous terrestrial and aquatic systems.

*P. putida* KT2440 specifies a large number of energy-gated outer membrane channels for substrates coupled to lower affinity cytoplasmic membrane transporters, a combination related to the ability of Proteobacteria to thrive in dilute aquatic environments (Niermman et al., 2001). *P. putida* KT2440 has 29 genes that share a TonB-dependent receptor C-terminal region, whereas bacteria such as species of Enterobacteria, which inhabit nutrient-rich niches, usually will have less than 10 (*Caulobacter crescentus* strain CB15 has 46), and a large number of high affinity transporters. The large majority of these TonB-

dependent receptors seem to be associated with iron uptake.

In addition to these diverse transport systems, *P. putida* KT2440 possesses a complex repertoire of chemosensory systems for detecting and responding to environmental signals (two-component sensors, sensor histidine kinases, chemotaxis, flagellum-related genes, etc.), and 17 extracytoplasmic function (ECF) sigma factors, 13 of which are associated with subfamily protein signature. Other “environmental” bacteria also have large numbers of ECF sigma factors: *P. aeruginosa* strain PAO has 17, *C. crescentus* 13, *Mesorhizobium loti* 19, *Sinorhizobium meliloti* 11, and *Agrobacterium tumefaciens* 11, although most genomes sequenced to date have far fewer. Furthermore, like *P. aeruginosa* strain PAO1, *P. putida* KT2440 has 22  $\sigma^{54}$ -dependent transcriptional regulators, which is more than what is observed in most bacterial genomes thus far sequenced, including those of plant-associated bacteria.

**SOIL AND PLANT ENVIRONMENTS.** Genome features associated with a soil lifestyle include a 1-acyl-*sn*-glycerol-3-phosphate-synthase that would form N-(3-hydroxy-7-*cis*-tetradecenoyl) homoserine lactone, a quorum-sensing molecule, and a large battery of stress response systems appropriate to the fluctuating and adverse environmental conditions of terrestrial habitats. These include active efflux systems for metals (arsenate, arsenite, copper, cadmium, chromate, cyanate, etc.), organic solvents (toluene), paraquat (two paralogous sets), antibiotics (e.g., penicillin) and, interestingly, the export of fusaric acid, a toxin produced by common fungal phytopathogens such as *Fusarium oxysporum* (Schnider-Keel et al., 2000), and several export systems for biopolymers and antibiotics. These characteristics underscore the potential of *P. putida* for biocontrol activity against fungal pathogens of plants. At least, one ABC efflux transporter shows similarities to ABC pumps associated with the efflux of organic solvents (Kim et al., 1998).

Interestingly, the *P. putida* KT2440 genome possesses the determinants for resistance against many heavy metals, as this capacity has not been recognized to any great extent in *Pseudomonas*. Additionally, not less than 13 putative glutathione-S-transferase genes were identified in the *P. putida* KT2440 genome. This number is similar to those in other *Pseudomonas* (i.e., *P. aeruginosa* [15] and *P. fluorescens* [10]), or in *C. crescentus* (19), *M. loti* (10) and *S. meliloti* (16), although it is much larger than those of most sequenced genomes. Glutathione-S-transferases generally act on synthetic chemicals (Vuilleumier, 2001), although these proteins are also known to play a significant role in the deg-

radation or inactivation of electrophilic compounds, such as most xenobiotics and heavy metals (Fraser et al., 2002).

A major stress factor in soils is water deprivation, and several genes (including those for flagellin, flagellar hook, two-component sensor, outer membrane protein, sugar binding proteins, peptidyl-prolyl *cis-trans* isomerases, two-component regulators, etc.) have been observed to be involved in water deprivation stress in *P. putida* KT2440. In fact, the response of *P. putida* KT2440 to water stress seems to involve the differential expression of membrane proteins, flagella related proteins, enzymes with action on membrane composition, or heat shock proteins. *P. putida* KT2440 has a large number (10) of genes coding for universal stress proteins, which is similar to that of *Halobacter* (11) and *P. aeruginosa* (8), but higher than in most other bacterial genomes sequenced thus far. Furthermore, *P. putida* KT2440 has genes for 7 cold shock proteins, similar to *P. aeruginosa* (6) and *M. loti* (7), and more than most other bacteria. *P. putida* KT2440 has determinants for 5 heat shock proteins, *M. loti* has 7, whereas *P. aeruginosa* and *C. crescentus* each have 2. *P. putida* KT2440 possesses a number of stringent starvation-related proteins (15), most of which (13) are glutathione-S-transferases (*P. aeruginosa* has 18, *M. loti* has 10, and *C. crescentus* has 19), whereas in most other genomes, the numbers of starvation-related proteins are substantially lower.

**RHIZOSPHERE AND BIOFILM FITNESS** *P. putida* KT2440 has been shown to be a versatile, aggressive colonizer, being able to establish itself and persist within the rhizosphere and in bulk soils at high cell densities (Molina et al., 2000). Features that contribute to rhizosphere and soil fitness include adhesion and colonization abilities, antibiotic production, resistance to (multiple) antibiotics, capability to use seed and root exudates, production and utilization of siderophores, and ability to cope with oxidative stresses.

**ADHESION.** *Pseudomonas putida* KT2440, other *P. putida* strains, as well as *P. fluorescens* and other *Pseudomonas* species, colonize and quickly proliferate in the rhizosphere of a number of agriculturally important plants. The *P. putida* KT2440 genome has three relatively large ORFs, encoding the largest protein in the genome (8682 amino acids), which has been demonstrated to be essential for seed colonization in *P. putida* (Espinosa-Urgel et al., 2000). The presence of an operon for the biosynthesis of cellulose (absent in *P. aeruginosa*), as in *A. tumefaciens* and in rhizobia suggests that cellulose may facilitate the



adhesion of *P. putida* KT2440 to root tissue (Mathysse et al., 1983).

Two previously uncharacterized polysaccharide biosynthesis and export gene clusters were also identified in *P. putida* KT2440. The first cluster, absent from the genome of *P. aeruginosa*, includes four putative glycosyl transferases, a putative serine *O*-acetyltransferase, and a VirK homologue, a factor essential for intercellular spreading of *Shigella*. The second cluster included, as well, four putative glycosyl transferases, two putative lipopolysaccharide core biosynthesis proteins, and a deoxy-D-manno-octulosonic-acid (KDO) transferase. Polysaccharides are likely to play a role in the attachment of *P. putida* to plant roots. Other surface polysaccharides and lipopolysaccharides and cell-envelope components, as well, are important for adhesion to plant surfaces, as shown by Sauer and Camper (2001), who detected upregulation of genes involved in the biosynthesis of exo- and lipopolysaccharides (EPS and LPS, respectively) as well as in membrane protein genes in *P. putida* following attachment.

In the *P. putida* KT2440 genome, 23 of the 24 genes for alginate biosynthesis or regulation have been identified. The omission of the gene, the fine-tuning transcriptional regulator AlgM/MucC, is likely to hamper alginate biosynthesis (Ohman et al., 1996). Previous studies suggest that alginate biosynthesis in *P. putida* may be relatively common and it cannot be ruled out that *P. putida* KT2440 is not able to produce alginate under appropriate conditions, for instance upon attachment to plants.

**COLONIZATION AND DEFENSE.** Effective colonization requires the specific action and coordination of certain enzymes and proteins. Gene clusters in *P. putida* KT2440, encoding several proteins specifically involved in plant-rhizosphere interactions are absent in *P. aeruginosa* PAO1, for example, a site-specific recombinase that is essential for competitive root colonization in *P. fluorescens* (Dekkers et al., 1998). Interestingly, *P. putida* KT2440 not only colonizes roots easily, but it actually senses metabolites in the neighborhood of the rhizosphere and moves quickly towards the roots (Espinosa-Urgel et al., 2002). In addition to a complex chemosensory and chemotactic machinery (Nelson et al., 2002), *P. putida* KT2440 has a type IV fimbrial biogenesis gene set, a large operon for the biosynthesis of flagella as well as the genes coding for type I pili, curli fiber, and two chaperone/usher gene clusters, all of which are likely to contribute to the attachment capacity of *P. putida* KT2440. Consistent with this, Sauer and Camper (2001) reported that various fimbrial biosynthesis and flagellar genes in *P. putida* were differentially expressed following

attachment onto surfaces. Several other studies have demonstrated the key role of motility-related proteins in the attachment and colonization of plant or inert surfaces by *P. putida* (Turnbull et al., 2001). Other proteins related to the general type II secretion pathway, such as those encoded by the genes involved in the transport and assembly of type IV pili, also have been shown to be important for adhesion of *P. putida* (Sauer et al., 2001).

The genome of *P. putida* KT2440 includes ORFs with similarities to genes for the synthesis of the lipodepsinonepeptide class phytotoxins, such as syringomycin, as well as a cluster containing genes for polyketide biosynthesis that are typically found in antibiotic-producing strains of *Pseudomonas* species. *P. aeruginosa* pyocins (bacteriocins) have been shown to have bactericidal activity towards related pseudomonads, as well as other bacteria. Note that *P. putida* KT2440 has a large number of pyocin-related genes, 24 of them orthologues to those in *P. aeruginosa* PAO1 (Nelson et al., 2002). Additionally, *P. putida* KT2440 and other *P. putida* strains are protected from other bacterial strains or a plant host by extruding toxic compounds through their many multidrug efflux systems and by synthesis of enzymes that include penicillin resistance proteins, (metallo)- $\beta$ -lactamases, cardiolipin proteases, phospholipase D, endolysin, etc. In many cases, this response is surface induced (Espinosa-Urgel et al., 2000; Sauer and Camper, 2001).

*P. putida* KT2440 exhibits extensive transport capabilities, with approximately 350 cytoplasmic membrane transport systems, 15% more than *P. aeruginosa* PAO1. The genes encoding for these systems form approximately 12% of the whole genome. The largest family corresponds to an ABC transporter (94 paralogous members), of which a significant proportion is predicted to be devoted to amino acid uptake. This is consistent with the ability for *P. putida* KT2440 to colonize plant roots, since root exudates are rich in amino acids, and reflects its physiological emphasis on the metabolism of amino acids and their derivatives to compete successfully in the rhizosphere. *P. putida* KT2440 also has the determinants for 11 LysE family amino acid efflux transporters (*P. aeruginosa* PAO1 has only one) that, presumably, play a key role in preventing the accumulation of inhibitory levels of amino acids or their analogues into the cell. Also consistent with its ability to colonize plant roots, *P. putida* KT2440 has a predicted ABC family opine transporter (previously been described for other rhizosphere bacteria; Lyi et al., 1999) and enzymes for the metabolism of opines, suggesting that it is capable of exploiting plant-produced opines induced in the rhizosphere by other bacterial species.

Opines are amino acids found in hairy-root hyperplasia and crown-gall tumors (Dessaux et al., 1993), and are important C- and N-sources for promoting rhizosphere-associated growth of Gram-negative bacteria.

In addition to these transport systems, the genome contains the determinants for the import of sugars (e.g., glucose, ribose and fructose), organic acids (aromatic, dicarboxylic and tricarboxylic), oligopeptides, anions (taurine, phosphate, phosphonate, sulfate, sulfonate and nitrate) and cations (ammonium, magnesium, copper, zinc, nickel, cobalt, potassium, sodium, heme and iron). Interestingly, *P. putida* KT2440 has only one phosphotransferase system (PTS) sugar transporter and a PTS-like system for nitrogen. Another large (i.e., the second largest) class of transporters is that of the Major Facilitator Superfamily, for which 48 determinants were found in the genome of *P. putida* KT2440. Most of the members are predicted to be involved in the efflux of toxic compounds although, in many cases, the substrate is unknown. The same applies to another large class, the resistance-nodulation-cell division (RND) efflux transporters.

**IRON UPTAKE.** A major phytoprotective effect of fluorescent pseudomonads is thought to be due to the production of siderophores under iron-limiting conditions, as well as to the ability to capture iron more efficiently than do plant pathogens (O'Sullivan and O'Gara, 1992; Walsh et al., 2001). Accordingly, *P. putida* KT2440 produces a siderophore, pyoverdine, which is a complex polypeptide produced by most fluorescent species of *Pseudomonas* and is strain-specific (Meyer et al., 2002). The pyoverdine genes in *P. putida* KT2440 are clustered in three groups and the organization is similar to that in the phytoprotectant bacteria *P. fluorescens* strain SBW25 and *P. putida* strain WCS358. The main pyoverdine transcriptional activator gene, *prfI*, is located next to the pyoverdine synthetase gene and is divergently transcribed. The gene in *P. putida* KT2440 has 93% identity with *prfI* experimentally characterized in *P. putida* WCS358 (Venturi et al., 1995) and 85% identity with *pvdS*, which is involved in the regulation of various pyoverdine biosynthesis genes in *P. aeruginosa* (Visca et al., 2002). The expression of the genes for pyoverdine production and siderophore uptake in the rhizosphere is influenced by other bacteria that coexist with *P. putida* (Loper and Henkels, 1999). Consistent with this, *P. putida* KT2440 has 29 genes whose products are predicted to be TonB-dependent outer-membrane siderophore receptors, most of which are within a gene cluster involving a transmembrane sensor, an ECF  $\sigma^{70}$  factor, and a transcriptional regulator, as predicted by Visca et al. (2002) for various

prokaryotes. In some cases, the siderophore receptors are clustered next to a siderophore transport system or siderophore biosynthesis genes, as for pyoverdine biosynthesis. Not surprisingly, the number of siderophore receptors in *P. putida* KT2440 is similar to that in the phytoprotectant *P. fluorescens* SBW25 but much larger than that in plant pathogens *P. syringae*, *Xylella fastidiosa*, *Ralstonia solanacearum* or *Agrobacterium tumefaciens*.

**OXYGEN METABOLISM.** *P. putida* KT2440, similar to plant symbionts *S. meliloti* and *M. loti* and the phytoprotectant saprophyte, *P. fluorescens*, has two complete sets of cytochrome *c* oxidase of the type *cbb3*, which has a high affinity for oxygen. Plant pathogens such as *P. syringae* or *A. tumefaciens* have only one set, and *X. fastidiosa* and *R. solanacearum* have none. Additionally, both *P. putida* KT2440 and *S. meliloti* have a second (*S. meliloti* has a third) formate dehydrogenase, which are important in fermentative processes. These two observations are consistent with the need of these bacteria to survive in the low-oxygen conditions of the rhizosphere or plant nodules.

To be able to thrive in their hosts, plant-associated microorganisms (both pathogens and symbionts or mutualists) must cope with oxidative stress, which is the first line of defense by plants against intruders. Not surprisingly and similar to other plant-associated microorganisms, *P. putida* KT2440 has many genes for dealing with oxidative stresses, including superoxide dismutases, catalases, (betaine-) aldehyde dehydrogenases, etc. In some cases, the regulation of these genes is iron-dependent and coupled to that of iron-binding proteins such as bacterioferritin.

Other relevant findings in *P. putida* KT2440 are the determinants for a bacteriophytochrome photoreceptor system that, in related prokaryotes, binds biliverdin, a breakdown product of heme metabolism (Bhoo et al., 2001). Although their precise functions are not fully known, it has been suggested such bacteriophytochromes play a role in signal transduction under oxygen and iron limitation. Interestingly, in prokaryotes, these phytochromes have been found only in microorganisms whose main habitats are soils and plants, which may be indicative of a habitat-specific function.

**POTENTIAL PATHOGENICITY FACTORS.** In addition to the genes described above, *P. putida* KT2440 has a number of genes that have been considered to be involved in animal or plant pathogenesis. ORFs coding for *gacA*, a cognate response regulator of *gacS* (sensor kinase) and a global regulator of secondary metabolites in Gram-negative bacteria are found also in *P. putida* KT2440. These genes are

involved in the regulation of the syringomicin production in *P. syringae* and of virulence functions in *P. aeruginosa* PAO1 (Rhame et al., 2001). Also of interest is the presence of determinants for several thiol:disulfide bond-forming enzymes, of which some have been shown to be involved in pathogenesis of both human and plant pathogens (Cao et al., 2001).

*P. putida* KT2440 possesses two orthologues to the *htrA* serine protease family, which have been shown to be involved in oxidative stress responses in pathogenic strains of *Salmonella* and *Yersinia* species. Additionally, one ORF encodes for a *marR* family regulator similar to *rovA* of *Y. pestis* and *Y. pseudotuberculosis*, which, in these bacteria, mediates the regulation of invasion in response to environmental signals.

Membrane lipopolysaccharides (LPS), particularly the B-band of O antigen, are often associated with virulence by many pathogens, including *P. aeruginosa* (Belanger et al., 1999). *P. putida* KT2440 has a large number of genes coding for surface polysaccharides and lipopolysaccharides, including those for the synthesis of the B-band of O antigen. However, the roles of these genes in pathogenicity are questionable.

Finally, several other ORFs, which seem to encode reported pathogenic factors have been detected: 1 ankyrin domain protein, 1 hemolysin-type calcium-binding bacteriocin, 7 leucine-rich domains, 1 endoglucanase, 3 phospholipase Ds, 7 lytic transglycosylases,  $\beta$ -metallo-lactamases, and VGR proteins (i.e., proteins with repetitions of Val-Gly). Interestingly, in the genome of *P. putida* KT2440, 23 paralogs of the *hlyD* (hemolysin) family secretion protein are found. However, still unclear is whether these proteins are expressed or, if so, whether they are associated with pathogenesis or simply have a habitat-specific function.

**CENTRAL METABOLISM** Like many other pseudomonads and rhizosymbionts, *P. putida* KT2440 has an incomplete glycolytic pathway, lacking 6-phosphofructokinase, and uses a complete Entner-Doudoroff route for utilizing glucose and other hexoses. However, it does have the genes coding for a fructose-1,6-biphosphatase and for glucose-6-phosphate isomerase, which are involved in early stages of gluconeogenesis, as well as in EPS biosynthesis. Also, like *P. aeruginosa* PAO1, *P. putida* KT2440 lacks the aldose-1-epimerase and glucose-1-phosphatase. Glucose is converted to glyceraldehyde-3-phosphate and pyruvate via the Entner-Doudoroff pathway, in which 6-phosphogluconate is a key intermediate. There are two main routes for converting glucose to 6-phosphogluconate: 1) by direct oxidation of

glucose into gluconate or 2-ketogluconate, both of which are transported to the cytoplasm, converted to 6-phosphogluconate and then further oxidized in the Entner-Doudoroff pathway; and 2) transport of glucose into the cytoplasm (via a sugar ABC-transporter), followed by phosphorylation of glucose to glucose-6-phosphate and then to 6-phosphogluconate. These two routes are cooperative, with the operation of one able to compensate for the loss of the other, although one or the other prevails, depending upon the physiological conditions. The direct oxidative pathway seems to prevail under aerobic conditions or higher substrate availability, whereas the phosphorylative pathway predominates under oxygen or glucose-limited conditions (Lessie and Phibbs, 1984).

The determinant for the membrane-associated pyrroloquinoline quinone (PQQ) glucose dehydrogenase is clustered with those for a porin B and for a TonB-dependent outer membrane receptor. The gluconokinase gene is just next to that for gluconate transport. Similarly, the genes encoding 2-ketogluconate metabolism (2-ketogluconate kinase, 2-ketogluconate 6-phosphate reductase, and epimerase) are clustered with those for a 2-ketogluconate transporter and a transcriptional regulator. Interestingly, this cluster is separated from that of the membrane-associated gluconate dehydrogenase complex, whose protein products convert gluconate to 2-ketogluconate by a transposase reading in the same direction and a potential terminator.

A patchy configuration of the various clusters coding for the enzymes of the initial stages of glucose utilization and the presence of different regulators in these gene clusters suggests a complex interplay of regulatory mechanisms with various control loops. Many of these regulatory mechanisms have been experimentally elucidated in various species of *Pseudomonas* (Clarke and Ornston, 1975), although a more comprehensive view of the gene organization within a genomic context has only become possible with the genome sequence information.

As in other *Pseudomonas* species, fructose metabolism in *P. putida* KT2440 occurs via a phosphoenolpyruvate phosphotransferase system (PTS). The genes are clustered with the 1-phosphofructokinase and the fructose transport system repressor. Other PTS-like systems encode proteins participating in a phosphorylation cascade and play an essential role in the regulation of nitrogen and carbon metabolism (Reizer et al., 1999).

*P. putida* KT2440 has also the glutathione-dependent glyoxalase I and II genes (as well as one for a D-lactate dehydrogenase) that code for the enzymes in the catabolism of methylglyoxal

to pyruvate. Several determinants for biosynthesis and catabolism of glycogen are clustered together within the genome. The phosphoglucomutase gene is found elsewhere in the genome and forms a cluster with an outer membrane ferric siderophore receptor, a transmembrane sensor, and an ECF  $\sigma^{70}$ , which indicates an iron-dependent regulatory link between environmental signals and glycogen metabolism. This is consistent with sensitive control of glycogen synthesis or for glycogen degradation, depending upon changing nutritional conditions.

Additionally, scattered throughout the genome are the genes for a periplasmic  $\beta$ -glucosidase, as well as those for  $\beta$ -(1-6)-glucan synthase,  $\beta$ -(1,3)-glucosyl transferase, and another copy of a glycosyl hydrolase. The  $\beta$ -(1-6)-glucan synthase and  $\beta$ -(1-3)-glucosyl transferase are similar to those in *Bradyrhizobium japonicum*, where they are involved in the synthesis of  $\beta$ -cyclic glucan, which is thought to be important for symbiotic interactions and to play a role in hypoosmotic adaptation (Bhagwat et al., 1996). The presence of genes coding for enzymes involved in the biosynthesis and degradation of other polysaccharides suggests a tight coupling to other "metabolic modules" and suggests a dependency upon prevailing environmental conditions.

All genes for the pentose phosphate pathway, the TCA cycle, the glyoxylate shunt, as well those for the oxidative and electron transport chain, are present in the genome of *P. putida* KT2440. ATP synthesis is driven by the resulting chemiosmotic gradient and occurs via an F-type ATP synthase. Analysis of the genome suggests that nitrogen and energy are derived from the catabolism of amino acids alanine, arginine, aspartate, asparagine, glutamine, glutamate, glycine, histidine, leucine, isoleucine, methionine, phenylalanine, serine, valine, lysine, proline, and hydroxyproline, and from the metabolism of a range of carbon compounds, including acetoin, fructose, butyrate, betaine, glucose, gluconate, glutarate, glycerol, hydantoin, lactate, malate, mannose, ribose, sorbate and sucrose, among others. Growth on these compounds has been confirmed experimentally by growing *P. putida* KT2440 on minimal media supplemented with these compounds.

**BIOSYNTHESIS** The genes for the synthesis of all 20 amino acids have been identified in the genome of *P. putida* KT2440. The first step in methionine biosynthesis from homoserine in *P. putida* KT2440 and other species of *Pseudomonas* is catalyzed by gene products with similarities to several known and putative homoserine *O*-acetyltransferases (Andersen et al., 1998;

Alaminos and Ramos, 2001). The presence of homologues to methionine biosynthesis genes suggests that *P. putida* KT2440 is able to generate homocysteine from *O*-succinylhomoserine through transsulfuration (via cystathionine) or by direct sulfhydrylation. Conversion of homocysteine to methionine is mediated by either of the two methionine synthetases present. The pathway configuration, which combines two different pathways, renders a greater flexibility to methionine biosynthesis and is consistent with studies demonstrating that methionine metabolism plays a crucial role in plant-microbe interactions and in bacterial fitness under stressful environmental conditions (Andersen et al., 1998; Tate et al., 1999). Genome comparisons reveal that this combination of pathways is conserved in soil or plant-associated Gram-negative bacteria (*P. syringae*, *P. fluorescens*, *M. loti*, *B. fungorum*, *R. solanacearum* and *R. metallidurans*) and *X. fastidiosa*.

The pathways for the synthesis of purines, pyrimidines and nucleotides are all complete in the genome of *P. putida* KT2440. *P. putida* KT2440 can synthesize and elongate fatty acids from acetate. The genes for at least two  $\beta$ -oxidation pathways are present. According to its genome content, *P. putida* KT2440 should be able to synthesize an extensive variety of cofactors and prosthetic groups, including biotin, folic acid, ubiquinone, pyochelin, pantothenate, coenzyme A, ubiquinone, glutathione, thioredoxin, riboflavin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), NAD phosphate (NADP), porphyrin, thiamine, cobalamin, pyridoxal 5'-phosphate, tetrahydrofolate, and lipoate. In most cases, the genes encoding for the enzymes involved are scattered throughout the genome and do not aggregate in gene clusters. Although *P. putida* KT2440 is not known to grow under anaerobic conditions nor to denitrify, it has several determinants characteristic of metabolism under low oxygen tension, such as two oxygen-independent coproporphyrinogen III oxidase genes, a nitrite reductase complex, and several determinants typical of fermentative metabolism such as those for D-lactate dehydrogenase, phosphotransacetylase, formaldehyde dehydrogenase, and an acetoin gene cluster.

**SURFACE POLYMERS** More than 330 genes were found in the genome of *P. putida* KT2440 that are presumed to encode for proteins related to surface components and the biosynthesis and degradation of key cell-surface components. Apart from being an integral part of the cell structure, cell surface components are crucial for *P. putida* in determining motility and sensing and

in resistance and promoting attachment. The genes for the murein sacculus are grouped in three clusters scattered throughout the chromosome. Interestingly, two of these clusters include a penicillin-binding protein gene. The two other penicillin-binding protein genes are, as well, clustered with lipoproteins. The murein cluster includes also a group of genes for the synthesis of lipoic acid. Cell-division protein determinants are adjacent to a peptidoglycan synthesis gene and clustered with a  $\sigma^{32}$  factor. These patterns are conserved in *P. aeruginosa*, *E. coli*, *C. crescentus*, *R. solanacearum* and *A. tumefaciens*, and may reflect the close link between cell division and murein sacculus biosynthesis.

The LPS layer of the outer membrane in Gram-negative bacteria consists of a hydrophobic lipid A region anchoring the molecule in the outer leaflet, an oligosaccharide core, and an O-antigen (O polysaccharide; Raetz and Whitfield, 2002). The genes for lipid A synthesis are clustered together, and include genes involved in phospholipid biosynthesis and a gene coding for an outer membrane protein of the bacterial surface antigen family. This cluster-like structure is conserved in *P. aeruginosa*, *E. coli*, *C. crescentus* and *S. meliloti*, which agrees with the general observation that the A-lipid part of the LPS is more or less conserved within the Proteobacteria, whereas the O-antigen region (the immunogenic part in pathogens) tends to be variable, even within strains of the same species (Raymond et al., 2002).

The oligosaccharide core can be divided, conceptually, into two regions: inner core (lipid A proximal) and an outer core. As in *P. aeruginosa* and *E. coli*, several of the inner core genes in *P. putida* KT2440 are clustered together in close proximity and include the determinants for the heptose kinases, the heptosyltransferases I and II, and two lipopolysaccharide core biosynthesis proteins. However, unlike *P. aeruginosa* PAO1, the 3-deoxy-D-manno-octulosonic-acid transferase gene and the ABC lipid transporter that exports lipid A and phospholipids into the outer membrane are not within this cluster but, instead, cluster together in a region containing other genes related to the LPS-EPS core biosynthesis. This cluster includes a toluene tolerance protein as well as other genes related to oligosaccharide core biosynthesis in an arrangement similar to that of *P. aeruginosa* PAO1.

The O-antigen region in strains of *P. aeruginosa* is the most variable part of the LPS and is responsible for the different immunogenic responses in eukaryotic hosts. It generally contains an A- and a B-band. Junker et al. (2001) have determined that *P. putida* KT2440 lacks the A-band, although the genes encoding proteins for its biosynthesis are within the genome. Some

of these genes, which code for various glycosyltransferases involved in the assembly of the A-band, are contained within a gene cluster that contains also the determinants for GDP-D-rhamnose biosynthesis from GDP-D-mannose and the determinants for an ABC transport system. This structure resembles in part that of *E. coli*, *Salmonella* or *P. aeruginosa* (Rochetta et al., 1998). However, this gene cluster in *P. putida* KT2440 lacks the gene encoding an essential rhamnosyl transferase and other genes that encode for proteins involved in B-band biosynthesis. Furthermore, the ABC system of *P. putida* KT2440 does not encode for the system responsible for the transport of O-repeat units, as in *P. aeruginosa* PAO1, but rather encodes for what seems to be a protein efflux system.

Adjacent to this A-band/B-band gene cluster, although reading in the opposite direction, is a cluster containing a cytidyl-CMP-*N*-acetylneuraminic acid synthetase gene next to a hydrolase of the haloacid-dehalogenase family plus two glycosyl transferases that are likely to be involved in O-antigen synthesis. Adjacent to this group is a cluster containing the genes encoding the enzymes for the synthesis of dTDP-L-rhamnose, a sugar nucleotide precursor essential for the assemblage of the A-band of the O-antigen, as well as for alginate formation (Rochetta et al., 1999). This cluster includes, as well, genes encoding a glycosyl transferase and an *O*-acetyltransferase involved in the B-band biosynthesis. Next to this gene cluster is another one that contains several genes encoding proteins possibly involved in the synthesis and transport of the A-lipid. These genes include the determinants for a mannosyl transferase, a phosphomannose isomerase, a phosphomannomutase gene, a lipopolysaccharide ABC-export system similar to the system for export of the individual and PP-linked O units (Raetz and Whitfield, 2002), a putative penicillin-binding protein (domain), a metallo- $\beta$ -lactamase protein, and a  $\beta$ -subunit of an integration host factor. Like other plant- and soil-associated bacteria, *P. putida* KT2440 is observed to lack the pathway for transport of the O-units across the membrane. *P. aeruginosa*, on the other hand, and similar to *E. coli* and *Salmonella*, has two pathways. Possibly, these differences may account for the avirulence of *P. putida*, as compared to *P. aeruginosa*, in which the O-antigen of the various serotypes is considered an important virulence factor. The G+C content of this gene cluster (54.3%) is lower than that for the overall genome and is likely to have been acquired by gene transfer. All of these clusters with genes encoding proteins involved in the production of LPS-EPS components have a G+C content atypically low for *P. putida* and different from that of neighboring genes not involved in

biosynthesis of lipopolysaccharides. This suggests that acquisition of the LPS-EPS genes may have occurred independently, perhaps in clusters, as has been suggested for *X. campestris* (Vorhölter et al., 2001). The complete set of genes, as well as the organization of the synthesis of the O-antigen part of the LPS is different from that in *P. aeruginosa* in its various serotypes (Rocchetta et al., 1999; Raymond et al., 2002). Rather, the set of *P. putida* KT2440 resembles those in the genomes of other soil and plant associated bacteria, such as *R. solanacearum*, *X. campestris*, *M. loti* or *S. meliloti*, perhaps reflecting the common habitat and evolutionary conditions of these bacteria.

**SULFUR METABOLISM** The operon enabling growth on aromatic and aliphatic sulfonates is present in *P. putida* KT2440. The genome contains the genes encoding the metabolism of alkylsulfonates, alkansulfonate-thiosulfate and thiosulfate. Furthermore, it has three genes coding for proteins similar to desulfurization enzymes although it lacks DszB monooxygenase for full desulfurization (Gallardo et al., 1996). However, the two specialized pathways encoded by *asfRABC* and *atsSRBCA*, described in other *P. putida* strains (Kahnert et al., 2000), are missing.

**PERIPHERAL METABOLISM** Strains of *P. putida* metabolize a variety of unusual and sometimes toxic compounds, particularly chemically stable aromatic organic compounds, and are frequently the predominant microbes isolated from polluted environments. A number of the corresponding catabolic pathways, for example, those for toluene and xylenes (Worsey and Williams, 1975; Williams and Worsey, 1976), phenols (Wong et al., 1976), naphthalene (Dunn, 1973), camphor (Rheinwald, 1973), biphenyls (Starovoirov, 1985), and chloroaromatics (Reineke, 1998) tend to be encoded by transmissible, broad host range catabolic plasmids that readily transfer between bacteria, and by catabolic transposons that readily transfer within the cell from plasmids to chromosomal locations. The location of catabolic determinants on mobile elements that can move horizontally among *Pseudomonas* species and related Proteobacteria provides such bacteria with considerable evolutionary potential, in terms of the metabolic capacities they can acquire for exploiting novel nutritional opportunities (Harayama et al., 1993).

*P. putida* KT2440 lacks catabolic plasmids but is known to have a chromosomal pathway for the degradation of benzoate, via catechol and 3-oxoadipate. Genome sequence analysis of *P. putida* KT2440 has revealed genetic determinants for putative enzymes able to transform a

variety of other aromatic compounds. Several of these (ferulate, coniferyl- and coumaryl alcohols, aldehydes and acids, vanillate, *p*-coumarate, *p*-hydroxybenzoate and protocatechuate) are related to lignin, generated during decomposition of plant materials, and abundant in the rhizosphere. These compounds are, therefore, likely to constitute a carbon pool for rhizosphere associated microorganisms. Furthermore, these compounds have been shown to be important for signaling in gene expression between plants and some bacteria (Venturi et al., 1998). The general strategy exploited by *Pseudomonas* species for the degradation of different aromatic compounds is to modify their diverse structures to common intermediates that can be funneled into a limited number of central pathways (Dagley, 1971). In *P. putida* KT2440, for example, initial steps in the metabolism of ferulate, *p*-coumarate, mandelate and benzoate would be mediated by different enzymes (upper pathways), although all routes ultimately converge via protocatechuate (ferulate and *p*-coumarate) or catechol (mandelate and benzoate) to the common 3-oxoadipate (also known as  $\beta$ -keto adipate) pathway. Interestingly, the 3-oxoadipate pathway has been found almost exclusively in soil- and plant-associated bacteria (Harwood and Parales, 1996) and is, presumably, evolved in response to the large number of phenolic compounds synthesized by plants.

## Applications

*Pseudomonas* species are valuable agents for biotechnological applications, primarily because of the malleable genomes of the species, supporting a diverse range of metabolisms, and a propensity to accept genetic modifications. As a group, strains of various species of *Pseudomonas* are able to extract energy from a wide range of relatively inert compounds. Applications for *Pseudomonas* range from xenobiotic waste treatment and oil spill management (Timmis et al., 1994; Dejonghe et al., 2000), treatment of fossil fuels for improved quality (Bos et al., 1996; Foght et al., 1996), biocatalysis for the synthesis of various compounds (Zeyer et al., 1985; Olivera et al., 2001), and as agents of plant growth (Lugtenberg and Weger, 1992) and protection (Voisard et al., 1994; Walsh et al., 2001). Furthermore, because of the heterogeneity of the natural habitat of *Pseudomonas*, special requirements for growth and metabolic activity may not be necessary during biotechnological applications.

**BIOREMEDIATION: DEGRADATION OF CHLOROAROMATICS BY PSEUDOMONAS** Chlorinated hydrocarbons comprise a spectrum of compounds that are

of industrial and economic importance. The introduction of chlorine atom(s) into a hydrocarbon significantly influences its physicochemical and biochemical properties and the tendency for bioaccumulation and environmental persistence. *Pseudomonas* species are well known for their widespread occurrence in natural habitats and their ability to utilize a variety of organic compounds, including haloaromatics.

**THE CHLOROCATECHOL PATHWAY OF PSEUDOMONAS SPECIES.** The first observations of microorganisms degrading chloroaromatic hydrocarbons were made in the 1960s by the groups of Alexander and Evans (Bollag et al., 1968; Tiedje et al., 1969; Gaunt and Evans, 1971; Evans et al., 1981). The elucidation of a dominant degradation pathway for chloroaromatics was carried out using one of the first bacterial strains observed to be able to degrade chloroaromatics, *Pseudomonas* sp. strain B13 (Dorn et al., 1974). *Pseudomonas* sp. B13 is capable of mineralizing 3-chlorobenzoate via 3-chloro- and 4-chlorocatechol as central intermediates. Degradation is initiated by a chromosomally encoded benzoate dioxygenase, forming 3-chloro- and 5-chlorodihydrodihydroxybenzoate in a 2:1 ratio (Reineke and Knackmuss, 1978a), followed by dehydrogenation resulting in 3-chloro- and 4-chlorocatechol (Reineke and Knackmuss, 1978b). Chlorocatechols are further degraded by a specialized set of enzymes, the chlorocatechol *ortho*-cleavage pathway (Dorn and Knackmuss, 1978a; Dorn and Knackmuss, 1978b; Schmidt and Knackmuss, 1980a; Schmidt et al., 1980b; Kaschabek and Reineke, 1992). Ring-cleavage of chlorocatechol is performed by a broad specificity chlorocatechol 1,2-dioxygenase to produce the corresponding chloro-*cis*, *cis*-muconates (Tiedje et al., 1969; Dorn and Knackmuss, 1978a; Dorn and Knackmuss, 1978b; Schmidt et al., 1980b; Broderick and O'Halloran, 1991). The elimination of the chlorine substituent has been assumed to occur spontaneously after 2-chloro- and 3-chloro-*cis*, *cis*-muconate are converted by chloromuconate cycloisomerases to 5-chloro- and 4-chloromuconolactone, respectively (Schmidt and Knackmuss, 1980a). The dienelactones (*cis*-dienelactone formed from 3-chloro- and *trans*-dienelactone from 2-chloro-*cis*, *cis*-muconate) are converted by a dienelactone hydrolase into maleylacetate (Schmidt and Knackmuss, 1980a), and a maleylacetate reductase catalyzes the reduction of the double bond to form 3-oxoadipate, the common metabolite of the 3-oxoadipate and the chlorocatechol *ortho*-cleavage pathway (Kaschabek and Reineke, 1992).

The genetic background for chlorocatechol degradation in *Pseudomonas* has been elucidated using an independently isolated 3-

chlorobenzoate degrading *P. putida* strain AC858 (Chatterjee et al., 1981), and genes encoding chlorocatechol pathway enzymes were localized on a plasmid. Similarly, chlorocatechol genes were observed to be located on plasmids in various other chloroaromatic degrading isolates (Don, 1981), including *Pseudomonas* species (Van der Meer 1991). However, in the case of *Pseudomonas* sp. B13, the chlorocatechol genes were observed to be located on a 105-kb genomic island, named "the *clc* element," present in the chromosome in two separate locations (Ravatn et al., 1998). The *clc* element integrates into the 3'-end of a target glycine tRNA gene and obviously employs a phage-type integrase to achieve site-specific integrations (Ravatn et al., 1998; Van der Meer et al., 2001). With a certain frequency the element excises, resulting in a circular intermediate. In contrast to many pathogenicity islands, the *clc* element is self-transmissible to other recipient bacteria (Springael et al., 2002). The fact that chlorocatechol genes are localized on mobile genetic elements suggests that they are transferred readily and spread in the environment.

The genes encoding the chlorocatechol pathway usually form clusters, and only a few variations in the structures of the operons have been observed, in spite of the geographically distinct origins or differences in phylogenetic positions of the bacteria. Regulatory genes usually precede the structural genes and are orientated in the opposite direction. The gene products of the regulatory genes, which are members of the LysR family, act as positive regulators. 2-Chloro-*cis*, *cis*-muconate was demonstrated to be the inducer of the *clc* operon (McFall et al., 1997). However, not only is the structure of the operons of the chlorocatechol genes similar, but also iso-functional enzymes of the chlorocatechol pathway have been shown to be homologous and more similar to each other than to the corresponding enzymes of catechol pathways, indicating that the proteobacterial chlorocatechol pathways are derived from a common origin (Schlömman, 1994).

Like *Pseudomonas* sp. strain B13, various other *Pseudomonas* species have been described that degrade 3-chlorobenzoate via a chromosomally encoded benzoate dioxygenase/dehydrogenase system and chlorocatechol genes. Benzoate dioxygenase genes have been localized in *P. putida*, *P. aeruginosa* and *P. fluorescens*, and homologous genes seem to be spread among *Pseudomonas* species. In an early study, the capability to degrade benzoate was observed to be ubiquitously present in *P. aeruginosa*, *P. chlororaphis* and *P. putida*, but few *P. stutzeri* and *P. fluorescens* strains harbored that metabolic activity (Stanier et al., 1966). The substrate specificity

of benzoate dioxygenases is restricted usually to benzoate and 3-chloro-/3-methylbenzoate, as shown for chromosomally encoded enzymes from *Pseudomonas* sp. B13 (Reineke and Knackmuss, 1978a). Thus, strains, which have been obtained from enrichment cultures using 3-chlorobenzoate as the growth substrate, are usually the product of patchwork assembly, i.e., they harbor pathway segments from different origins obtained through horizontal natural gene transfer.

In contrast to benzoate dioxygenase, the toluate 1,2-dioxygenase (XylXYZ) of *P. putida* mt-2, whose natural function is the conversion of *m*- and *p*-toluate, transforms also 4-chlorobenzoate (Reineke and Knackmuss, 1978a). 4-Chlorobenzoate is a good substrate because of its structural analogy to *p*-toluate (4-methylbenzoate). Presumably, pathways evolved for the degradation of naturally occurring methyl-substituted growth substrates can often deal with substrates bearing chlorine substituents, since both substituents are of similar size.

Thus, it was reasoned that a combination of a toluate pathway with a chlorocatechol pathway should result in derivative strains capable of mineralizing 4-chlorobenzoate. *Pseudomonas* sp. B13 acquired the ability to utilize novel substrates like 4-chloro- and 3,5-dichlorobenzoate after transfer of the TOL plasmid of *P. putida* mt-2, harboring the toluate dioxygenase genes (Reineke and Knackmuss, 1979). Similarly, transfer of the *clc* element into *P. putida* strain mt-2 resulted in 4-chlorobenzoate-degrading derivatives (Reineke et al., 1982).

Additionally, various other enzyme systems, usually involved in the degradation of naturally occurring aromatics such as toluene, phenol, salicylate or aniline are rather unspecific and tolerate additional substituents on the substrate molecule. Thus, by the judicious combination of such peripheral enzyme systems and central chlorocatechol pathway segments in a suitable host organism, complete hybrid pathways have been produced for various mono- and disubstituted chloroaromatics (Pieper and Reineke, 2000), such as chlorinated benzenes (Oltmanns et al., 1988), phenols (Schwien and Schmidt, 1982), salicylates (Rubio et al., 1986), anilines (Latorre et al., 1984) and toluenes (Abril et al., 1989; Brinkmann and Reineke, 1992).

One of the best-described enzyme systems for the activation of benzene derivatives is the broad substrate specificity toluene dioxygenase of *P. putida* strain F1, which belongs to the toluene/biphenyl family of Rieske non-heme iron dioxygenases (Gibson and Parales, 2000). The enzyme system comprises an electron transport chain of ferredoxin reductase and ferredoxin,

which channels electrons from NADH onto the catalytically active oxygenase consisting of  $\alpha$ - and  $\beta$ -subunits (Yeh et al., 1977), with the  $\alpha$ -subunit being predominantly responsible for the substrate specificity (Beil et al., 1998). Toluene is oxidized to the respective *cis*-dihydrodiol (Gibson et al., 1970), which is subsequently dehydrogenated to 3-methylcatechol. This enzyme system also accepts chlorobenzene, *p*-chloro- or *p*-bromotoluene as substrates, which are oxidized to their dihydrodiols and dehydrogenated to the respective catechols (for a review of toluene dioxygenase-catalyzed conversions, see Hudlicky et al., 1999). Similarly, 1,4-dichlorobenzene degraders can easily be evolved by acquisition of chlorocatechol genes in toluene degrading strains (Oltmanns et al., 1988). Only a few microorganisms have been isolated thus far on the basis of their abilities to degrade 1,2-dichloro- (Haigler et al., 1988), 1,3-dichloro- (De Bont et al., 1986), or 1,4-dichlorobenzene (Schraa et al., 1986; Oltmanns et al., 1988). Obviously, their enzymes are adapted specifically for transformation of their respective growth substrate. *Pseudomonas* sp. strain P51, isolated on the basis of its ability to degrade 1,2,4-trichlorobenzene (Van der Meer et al., 1987), contains a broad-spectrum chlorobenzene dioxygenase capable of transforming all dichloro- and even 1,2,4-trichlorobenzene. The respective *icb* genes (Werlen et al., 1996) are very similar to the respective *tod* genes involved in toluene degradation by F1 (e.g., 89% identity in amino acid sequence of the respective  $\alpha$ -subunits), indicating that small changes in amino acid sequence have a significant influence on substrate specificity.

The first enzymes of the chlorobenzene degradation pathway of strain P51, the chlorobenzene dioxygenase and the *cis*-chlorobenzene dihydrodiol dehydrogenase, are encoded on a plasmid-located transposon (Werlen et al., 1996), with the chlorocatechol genes located on the same plasmid. High similarity suggested that the chlorobenzene dioxygenase and dehydrogenase originated from a toluene or benzene degradation pathway, probably by horizontal gene transfer, to form a functional chlorobenzene degradative pathway in combination with the chlorocatechol genes.

The dioxygenation of toluene is only one of five different aerobic pathways for initializing toluene degradation. The TOL plasmid pWW0 is known to encode not only the enzymes for the degradation of *m*- and *p*-toluate (see above) but the complete set of enzymes necessary for the degradation of *m*- and *p*-xylene via the respective methylbenzoates and of toluene via benzoate as intermediates (Worsey and Williams, 1975). The degree of transformation of chloro-



toluenes by xylene monooxygenase (XylMA) depends on the position of the chlorine substituent. The substrate analogs 3-chloro- and 4-chlorotoluene are transformed at high rates, whereas no or only low activities have been detected with other chlorotoluenes. Substituents in the *ortho* position impair substrate binding (Brinkmann and Reineke, 1992). Transfer of the TOL plasmid into strain B13 allowed the isolation of chlorotoluene-degrading *Pseudomonas* species (Abril et al., 1989; Brinkmann and Reineke, 1992).

Toluene monooxygenases that hydroxylate the aromatic nucleus at all three possible positions, producing 2-methyl-, 3-methyl-, or 4-methylphenol have been described (Schields et al., 1989; Whited and Gibson, 1991; Olsen et al., 1994) and include the xylene monooxygenase systems of *P. stutzeri* OX1 (Bertoni et al., 1996) and *P. mendocina* KR1 (Whited and Gibson, 1991). These enzymes belong to an evolutionarily related family of soluble di-iron hydroxylases, including also phenol hydroxylases and methane monooxygenases. The enzyme complexes consist of an electron transport system comprising a reductase (and in some cases a ferredoxin), and a catalytic effector protein (Powlowski et al., 1997). Only limited information is available on the transformation of chlorophenols formed by monooxygenation by multicomponent toluene hydroxylases.

Salicylate hydroxylase is a flavoprotein monooxygenase that catalyzes the conversion of salicylate to catechol. The enzyme was first purified from *P. putida* S1 (Yamamoto et al., 1965), and later from various other *Pseudomonas* and pseudomonad species, and cloned and sequenced from various sources (You et al., 1991; Lee et al., 1995; Bosch et al., 1999a; Jones et al., 2000), predominantly *Pseudomonas* species. Usually, salicylate hydroxylase is included in the naphthalene pathway, and the gene encoding salicylate hydroxylase is followed by genes encoding a *meta*-cleavage pathway. Different so-called "NAH" plasmids harboring those genes have been described from *Pseudomonas* strains (Cane and Williams, 1982; Yen and Gunsalus, 1982), but in contrast, *P. stutzeri* AN10 harbors chromosomally located *nah* genes (Rosselló-Mora et al., 1994). Moreover, variations in gene organization have been observed. *P. stutzeri* AN10, besides containing *nahG* encoding salicylate hydroxylase located in one transcriptional unit with the *meta*-cleavage pathway genes, contains a second gene encoding salicylate hydroxylase, *nahW*, which is situated outside, but in close proximity to, this transcriptional unit. Both, the *nahG* and *nahW* genes of *P. stutzeri* AN10 are induced and expressed upon incubation with salicylate. Such a gene organization seems to be

common in naphthalene-degrading *P. stutzeri* strains (Bosch et al., 1999b). Despite differences in gene organization and partially low similarity (NahW shares 23–25% amino acid sequence identity to other salicylate hydroxylases), most salicylate hydroxylases described thus far exhibit similar substrate ranges with significant activities against 4-chloro- and 5-chlorosalicylate, and a lower activity against 3-chlorosalicylate (Lehrbach et al., 1984; Rubio et al., 1986; Bosch et al., 1999b; Jones et al., 2000). However, recently a new group of three component salicylate 1-hydroxylases has been described, which consist of a hydroxylase component, a ferredoxin, and a ferredoxin reductase (Pinyakong et al., 2003), differing significantly in substrate specificity from previously analyzed single component salicylate 1-hydroxylases. Given the broad substrate-specificity of salicylate 1-hydroxylases, it is not astonishing that chlorosalicylate-mineralizing strains of *Pseudomonas* species could easily be obtained by combining a salicylate hydroxylase with a functioning chlorocatechol pathway (Lehrbach et al., 1984; Rubio et al., 1986). Genes similar to those encoding salicylate hydroxylase (ca. 25–30% amino acid sequence identity) have been localized in the genomes of *P. aeruginosa* strain PAO1 and *P. putida* strain KT2440, although *P. putida* strain KT2440 is reported not to grow on salicylate (Jimenez et al., 2002). Analyses have shown that only a few strains of *Pseudomonas* species are capable of mineralizing salicylate, whereas such a capability seems to be common in *Burkholderia cepacia* (Stanier et al., 1966).

**OXYGENOLYTIC DEHALOGENATION** Usually, dioxygenases activating the aromatic ring attack either at two unsubstituted carbon atoms, as observed for benzenetoluene dioxygenase, or at an unsubstituted and a carboxylated carbon atom, such as demonstrated by benzoatetoluene dioxygenases, giving rise to *cis*-dihydrodiols that are transformed further by dehydrogenases to give 1,2-diphenols. Some ring-activating dioxygenases can bring about the dehalogenation of haloaromatic compounds. Benzoate dioxygenase of strain B13 attacks 2-fluorobenzoate predominantly in a 1,6-fashion, giving rise to a dihydrodiol, which is rearomatized to 3-fluorocatechol. As the ring-cleavage product 2-fluoromuconate is not a substrate for proteobacterial (chloro)muconate cycloisomerases, strain B13 cannot grow on 2-fluorobenzoate. However, prolonged adaptation resulted in derivatives that grow on this substrate. Obviously, spontaneous mutants of the benzoate dioxygenase arose, which performed a 1,2-dioxygenation (Engesser et al., 1980), such that one of the *vic*-hydroxyl groups in the

*cis*-dihydrodiol is bound to the same carbon as the fluoro-substituent. From such an unstable *vic*-dihydrodiol, the fluoro-substituent will be spontaneously eliminated to give catechol. However, 2-chlorobenzoate cannot be used as a growth substrate by B13-derivatives, indicating that proteobacterial benzoate dioxygenases cannot accommodate such a voluminous substituent in the *ortho*-position.

Since the 1980s, bacteria capable of degrading 2-chlorobenzoate have been reported and, thus far, only *Pseudomonas* and *Burkholderia* species have been observed to possess such a capability. The isolates could be grouped into those capable of degrading 2-chlorobenzoate, such as *P. putida* strain CLB250 (Engesser and Schulte, 1989) and *P. aeruginosa* strain 2-BBZA (Higson and Focht, 1990), and those capable of degrading also 2,4-dichloro- or 2,5-dichlorobenzoate, such as *P. stutzeri* strain KS25 (Kozlovsky et al., 1993), *P. putida* strain P111 (Hernandez et al., 1991), *P. aeruginosa* strain JB2 (Hickey and Focht, 1990), and *P. aeruginosa* strain 142 (Romanov et al., 1993). All of these organisms catalyze a 1,2-dioxygenation of 2-chlorobenzoate, thereby forming catechol, whereas 4-chlorocatechol is produced by the dioxygenation of 2,4-dichloro- and 2,5-dichlorobenzoate. Inasmuch as the degradation of 4-chlorocatechol requires a chlorocatechol degradative pathway, presumably organisms capable of degrading 2-chlorobenzoate only, and those capable of degrading also 2,4-dichloro- or 2,5-dichlorobenzoate, differ by the presence of a chlorocatechol pathway. However, two distinct 2-chlorobenzoate degrading dioxygenase systems have been described. The two-component 2-halobenzoate 1,2-dioxygenases (oxygenase consisting of  $\alpha$ - and  $\beta$ -subunits and a reductase) are similar (56–59% in  $\alpha$ -subunits amino acid sequence identity) to a two-component toluate 1,2-dioxygenase and to benzoate and toluate 1,2-dioxygenases (Harayama et al., 1991; Cowles et al., 2000) and belong to the family of benzoate dioxygenases (Gibson and Parales, 2000). These 2-halobenzoate dioxygenases are characterized by their high activity against 2-chlorobenzoate (Fetzner et al. 1992; Suzuki et al., 2001) but negligible activity against 2,4-dichloro- or 2,5-dichlorobenzoate. In contrast, the 2-chlorobenzoate dioxygenase system of *P. aeruginosa* strain 142 is a three-component dioxygenase system (an oxygenase consisting of  $\alpha$ - and  $\beta$ -subunits, ferredoxin and reductase; Romanov and Hausinger, 1994). Moreover, the  $\alpha$ -subunits of 2-chlorobenzoate dioxygenases of *P. aeruginosa* strains JB2 and 142 (Tsoi et al., 1999) exhibit only 22% amino acid sequence identity with that of *B. cepacia* strain 2CBS but higher levels of sequence similarity (42%) with the

salicylate 5-hydroxylase from *Pseudomonas* sp. strain U2 (Fuenmayor et al., 1998). Thus, 2-chlorobenzoate dioxygenases are functionally similar but represent two different lineages with distinct activities.

**METABOLISM OF CHLOROAROMATICS BY 3-OXOADIPATE PATHWAY ENZYMES** The aerobic degradation of aromatic compounds usually involves the successive activation and modification such that they are channeled toward a few dihydroxylated intermediates, such as catechol, gentisate or protocatechuate, which are then subjected to ring cleavage. A variety of the enzyme systems capable of activating aromatic compounds are characterized by broad substrate specificities and transform chlorinated substrate analogues, often resulting in the formation of chlorinated catechols (see above). However, only a small fraction of bacteria able to transform chloroaromatics is also capable of mineralizing them, as this usually requires the chlorocatechol pathway. Chlorocatechols are regarded as environmentally important intermediates and their metabolic fate can be of environmental significance. The chromosomally encoded 3-oxoadipate pathway is one such pathway that is widely distributed in soil bacteria and fungi (Harwood and Parales, 1996).

Marked differences have recently been demonstrated between reactions catalyzed by the chlorocatechol and the 3-oxoadipate pathway enzymes. In both cases, chlorocatechols were subject to intradiol cleavage producing the corresponding *cis,cis*-muconates (Blasco et al., 1995). However, muconate and chloromuconate cycloisomerases perform distinct reactions. Whereas chloromuconate cycloisomerases catalyze a dehalogenation of 3-chloro-*cis,cis*-muconate to form *cis*-dienelactone, muconate cycloisomerases catalyze a dehalogenation and decarboxylation to form protoanemonin, a compound of high toxicity (Seegal and Holden, 1945). Protoanemonin formation was observed to be a common reaction performed by proteobacterial muconate cycloisomerases, including those from strains of *Pseudomonas* species (Vollmer et al., 1998). Protoanemonin formation (due to channeling of intermediary chlorobenzoate into the 3-oxoadipate pathway), in turn, was assumed to be the reason for the poor survival of organisms cometabolizing polychlorinated biphenyls in soil microcosms (Blasco et al., 1997). Even chloroprotoanemonin was reported to be formed from 2,4-dichloromuconate by *P. putida* muconate cycloisomerase (Kaulmann et al., 2001). Also in the case of 2-chloromuconate turnover, muconate and chloromuconate cycloisomerases were shown to catalyze different reactions. Whereas chloromuconate

cycloisomerase catalyzes dehalogenation to form *trans*-dienelactone, via 5-chloromuconolactone as an intermediate (Vollmer and Schlömann, 1995), muconate cycloisomerases catalyze cycloisomerization only, to form 2-chloro- and 5-chloromuconolactone as stable products (Vollmer et al., 1994). Only chloromuconate cycloisomerase, but not muconate cycloisomerase, catalyzes the dehalogenation of 5-chloromuconolactone (Vollmer et al., 1994; Vollmer and Schlömann, 1995). However, 5-chloromuconolactone has been shown to be a substrate of proteobacterial muconolactone isomerases (Prucha et al., 1996a; Prucha et al., 1996b) of the 3-oxoadipate pathway. Muconolactone isomerase catalyzes a dehalogenation of 5-chloromuconolactone to form predominantly *cis*-dienelactone. The proposed mechanism was via abstraction of the C4 proton followed by spontaneous chloride elimination. Like 5-chloromuconolactone, 2-chloromuconolactone also harbors a proton at C4, which can be abstracted by muconolactone isomerase, and protoanemonin was shown to be the reaction product (Skiba et al., 2002).

**THE ALTERNATIVE PATHWAY OF 4-HALOCATECHOL DEGRADATION IN *PSEUDOMONAS*** Muconate cycloisomerases, as described above, form protoanemonin as a toxic dead-end product (Blasco et al., 1995). Hence, transformation of 4-chlorocatechol by enzymes of the 3-oxoadipate pathway constitutes a problematic catabolic misrouting in bacterial communities (Blasco et al., 1997). No effective activity against protoanemonin has been characterized thus far, although a degradative pathway with protoanemonin as an intermediate was proposed for 4-chlorocatechol degradation by *Pseudomonas* sp. strain RW10 (Wittich et al., 1999). This organism was shown to have a 3-oxoadipate pathway but no chlorocatechol pathway, thus forming protoanemonin from 3-chloromuconate. *Pseudomonas* sp. strain MT1, the most predominant organism in a 4-membered community isolated by continuous culture enrichment on 4-chlorosalicylate as sole carbon source (Pelz et al., 1999), was similarly proposed to have a new 4-chlorocatechol degradative pathway, which was thought to involve protoanemonin as intermediate. Both strains do not express enzymes of the chlorocatechol pathway but contain a high level of a *trans*-dienelactone hydrolase when grown on chloroaromatics. This enzyme was shown to be the key enzyme of a novel 4-chlorocatechol degradative pathway operating in *Pseudomonas* sp. strain MT1 (Nikodem et al., 2003), and probably also in *Pseudomonas* sp. strain RW10. 4-Chlorocatechol was subject to ring-cleavage

by a catechol 1,2-dioxygenase to yield 3-chloromuconate. 3-Chloromuconate was transformed to protoanemonin as the dominant reaction product by muconate cycloisomerase of strain MT1. Formation of protoanemonin is obviously a dead-end product of the pathway. Even though *trans*-dienelactone hydrolase does not act on 3-chloromuconate nor on protoanemonin, the simultaneous presence of muconate cycloisomerase and *trans*-dienelactone hydrolase yields considerably smaller protoanemonin concentrations, although higher amounts of maleylacetate from 3-chloromuconate, than muconate cycloisomerase alone (Nikodem et al., 2003). It is suggested that this enzyme acts on 4-chloromuconolactone as an intermediate in the muconate cycloisomerase-catalyzed transformation of 3-chloromuconate, thus preventing protoanemonin formation in favor of maleylacetate formation. The formed maleylacetate is reduced subsequently by maleylacetate reductase. Chlorocatechol degradation in strain MT1 thus occurs by a new pathway consisting of a patchwork of reactions recognized from the 3-oxoadipate pathway (catechol 1,2-dioxygenase and muconate cycloisomerase), the chlorocatechol pathway (maleylacetate reductase), and a *trans*-dienelactone hydrolase.

**ALTERNATIVE CENTRAL PATHWAYS FOR CHLORINATED 1,2-DIPHENOLIC INTERMEDIATES** For quite a long time, the presence of a catechol *meta*-cleavage pathway was assumed to interfere significantly with degradation of chloroaromatics. Catechol *meta*-cleavage routes are widespread in *Pseudomonas* species and are usually involved in the degradation of methyl-substituted compounds, such as toluene or methylphenols (Bayly et al., 1966; Worsley and Williams, 1975; Zylstra et al., 1988; Schingler et al., 1992). One of the reasons for interference was assumed to be the formation of a suicide product, reactive acyl chloride, e.g., from 3-chlorocatechol by the catechol 2,3-dioxygenase of *P. putida* mt-2 (Bartels et al., 1984). The formation of acyl chloride results in irreversible inactivation of the ring cleavage enzyme. Other enzymes such as the catechol 2,3-dioxygenase of *P. putida* F1 were reported to be reversibly inactivated by 3-chlorocatechol. Inactivation has been attributed to the potential of the substrate to chelate the active site ferrous ion (Klecka and Gibson, 1981). In other cases, reversible inactivation was shown to be due to a rapid oxidation of the active site ferrous iron into its ferric form with concomitant loss of activity (Vaillancourt et al., 2002).

Recently, *P. putida* strain GJ31 was observed to degrade chlorobenzene rapidly via 3-chlorocatechol and using a *meta*-cleavage

pathway (Mars et al., 1997). In contrast to other catechol 2,3-dioxygenases, which are subject to inactivation, the chlorocatechol 2,3-dioxygenase of strain GJ31 converts 3-chlorocatechol (Kaschabek et al., 1998; Mars et al., 1999). Stoichiometric displacement of chloride during substrate turnover leads to the production of 2-hydroxyruinate, which is further converted through the *meta*-cleavage pathway.

In contrast to 3-chlorocatechol, 4-chlorocatechol is a moderate substrate for various catechol 2,3-dioxygenases (Murray et al., 1972; Bartels et al., 1984; Kitayama et al., 1996), among them catechol 2,3-dioxygenases of family I.2.A (Eltis and Bolin, 1996), which are involved in the degradation of methylaromatics by *Pseudomonas* species. Some chlorinated compounds, degraded via 4-chlorocatechol, have been postulated to be mineralized via a catechol-degrading *meta*-cleavage pathway (Janke and Fritsche, 1979; McCullar et al., 1994; Arensdorf and Focht, 1995; Hollender et al., 1997), although the manner in which the products are dechlorinated is unknown.

Protocatechuate is a central intermediate in the degradation of various carboxylated aromatic compounds. Three modes of further degradation of protocatechuate have been reported. The intradiol cleavage by a protocatechuate 3,4-dioxygenase seems to be widespread in *Pseudomonas* species (all strains of a collection of more than 100 strains of *Pseudomonas* species displayed such an activity; Stanier et al., 1966), and the locations of the respective *pca* gene clusters have been determined within the genomes of *P. aeruginosa* strain PAO1, *P. putida* strain KT2440, *P. fluorescens* strain Pfo-1, and *P. syringae* pv. tomato DC3000 (Jimenez et al., 2002). However, this pathway seems to be unsuited for the degradation of chloroprotocatechuate.

Whereas a protocatechuate 2,3-dioxygenase has so far been described only in an isolate of a *Bacillus* species (Wolgel et al., 1993), protocatechuate 4,5-dioxygenase seems to be widespread. Strains of *Delftia acidovorans*, *Comamonas testosteroni* and *Sphingomonas paucimobilis* usually exhibit such an activity (Stanier et al., 1966; Noda et al., 1990), whereas isolates of *Pseudomonas* species do not (Stanier et al., 1966).

However, genes similar to those encoding the protocatechuate 4,5-dioxygenase pathway were observed in the genome of *P. putida* strain KT2440, although the functioning of this pathway has not been proven yet (Jimenez et al., 2002). Only the protocatechuate 4,5-dioxygenase pathway has been shown to be functional for the degradation of chloroprotocatechuate, and the formation of 2-pyrone-4,6-dicarboxylate by

nucleophilic displacement of a halide ion from protocatechuates substituted with a halogen at the C5 of the nucleus was shown (Kersten et al., 1982; Kersten et al., 1985). This indicated that cyclization, entailing nucleophilic displacement of the halogen, provides an effective alternative to the enzyme suicide inactivation that occurs when a nucleophilic group of the dioxygenase undergoes acylation. Hydrolysis of the pyrone is followed by degradation through the *meta*-cleavage pathway.

**THE 4-CHLOROBENZOATE HYDROLYTIC DEHALOGENATION** The degradation of 4-chlorobenzoate by a pathway distinct from that involving chlorocatechol, but involving an early dehalogenation, was indicated as early as 1976 (Ruisinger et al., 1976). *Pseudomonas* sp. strain CBS3 can be regarded as the archetypal organism from which the metabolic route and the mechanism of hydrolytic dehalogenation have been elucidated in detail (Müller et al., 1984). The 4-chlorobenzoate dehalogenase system from *Pseudomonas* sp. strain CBS3 has been shown to be a three-component enzyme complex (Elsner et al., 1991; Löffler et al., 1991) and the role of each component has been clarified (Scholten et al., 1991; Savard et al., 1992). The activation of the substrate is carried out by an ATP-dependent 4-halobenzoate-coenzyme A ligase (Löffler and Müller, 1991; Chang et al., 1992). Identification of 4-chlorobenzoyl-coenzyme A, as an intermediate in the dehalogenation, is catalyzed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3 (Löffler et al., 1992). This is followed by dehalogenation, catalyzed by a 4-halobenzoyl-CoA dehalogenase, forming 4-hydroxybenzoyl-CoA (Liang et al., 1993; Löffler et al., 1995) in a hydrolytic substitution reaction. This enzyme was reported to share ancestry with the 2-enoyl-CoA hydratase family (Babbitt et al., 1992; Xiang et al., 1999). The last step in the reaction forming 4-hydroxybenzoate is carried out by the 4-hydroxybenzoate:coenzyme A thioesterase.

The genes encoding the 4-chlorobenzoate dehalogenase system were reported to be chromosomally localized and organized in an operon in the order: *fcB* (dehalogenase), *fcA* (ligase), and *fcC* (thioesterase; Babbitt et al., 1992). The gene organization in a second 4-chlorobenzoate-degrading *Pseudomonas* sp. strain DJ-12 was similar, although, with an additional three new genes, localized between *fcA* and C and, supposedly, responsible for 4-chlorobenzoate transport (Chae et al., 2000).

**METABOLISM OF CHLOROBIPHENYLS** Among chlorinated aromatics, the degradation of bicyclic compounds such as polychlorobiphenyls (PCBs)

or chlorinated dioxins has received special attention. A number of biphenyl-degrading organisms have been isolated and they are commonly capable of transforming PCB congeners. These organisms belong to Gram-negative and Gram-positive genera and include various isolates of *Pseudomonas* species, such as *P. pseudoalcaligenes* strain KF707, *P. putida* strain KF715, *P. putida* strain OU83, and catabolize biphenyl to benzoate and 2-hydroxypenta-2,4-dienoate via the so-called "upper pathway," consisting of four enzymes, i.e., biphenyl 2,3-dioxygenase (BphA), 2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase (BphB), 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD) (Boyle et al., 1992).

Biphenyl degradation genes usually are organized in gene clusters and have been localized on bacterial chromosomes (Furukawa and Miyazaki, 1986; Erikson and Mondello, 1992), plasmids (Hooper et al., 1989) and transposons (Springael et al., 1993). Organisms such as *P. putida* strain KF717 contain a *bphABCD* gene cluster. In *Burkholderia* sp. strain LB400, genes that encode enzymes involved in the transformation of the C-5 carbon released during hydrolysis of the ring-cleavage product to form benzoate and a glutathione S-transferase are located between *bphC* and *bphD* (Hofer et al., 1994). A detailed analysis of various biphenyl-degrading isolates revealed that such a genetic organization is not unique to *Burkholderia* sp. strain LB400 (Bartels et al., 1999) but occurs also in *P. pseudoalcaligenes* strain KF707 and various strains of *P. agarici*, *P. oleovorans*, *P. balearica* and *P. putida*, and seems to be widespread in *Pseudomonas*.

To a significant extent, the spectrum of PCB congeners that can be transformed by an organism is determined by the specificity of the biphenyl 2,3-dioxygenase, the enzyme which catalyzes the first step in the upper pathway and which belongs to the toluene/biphenyl family of Rieske type non-heme iron dioxygenases. Studies on various biphenyl 2,3-dioxygenases have revealed considerable differences in their congener selectivity patterns, as well as their preference of the attacked ring (Erikson and Mondello, 1993; Kimura et al., 1997; McKay et al., 1997; Seeger et al., 1999; Zielinski et al., 2002). The different substrate specificities in bacterial strains were due to relatively few differences in *bphA*, the gene coding the large subunit of the terminal biphenyl dioxygenase.

Although certain PCBs may serve as substrates for biphenyl dioxygenases, PCB-degrading organisms usually do not use PCBs as an energy source but rather cometabolize the substrates. Not surprisingly, metabolites of the

upper pathway may be formed as dead-end products (Furukawa et al., 1979; Bedard and Haberl, 1990; Seeger et al., 1997). Microorganisms capable of mineralizing a subset of PCB congeners have been obtained by judicious combination of pathway segments comprising the biphenyl upper pathway, a benzoate/toluate or 2-chlorobenzoate dioxygenase system and a chlorocatechol pathway (Mokross et al., 1990; Havel and Reineke, 1991; Hickey et al., 1992; McCullar et al., 1994; Hrywna et al., 1999), usually by *in vivo* conjugative mating of appropriate strains.

**TRANSFORMATION OF BIARYLETHERS** Studies by Cerniglia et al. (1979) and Klecka and Gibson (Klecka and Gibson, 1979; Klecka and Gibson, 1981), using naphthalene or biphenyl-degrading strains of *Pseudomonas* or *Sphingomonas* species, indicated that dibenzo-*p*-dioxin, dibenzofuran and chlorinated derivatives were transformed into dead-end products. Analysis of bacterial degradation of dibenzofuran (Engesser et al., 1989; Fortnagel et al., 1990), carbazole (Sato et al., 1997), dibenzo-*p*-dioxin (Wittich et al., 1992), and diphenyl ether (Schmidt et al., 1992; Dehmel et al., 1995) revealed a novel mode of dioxygenation reaction for the aromatic nucleus, termed "angular dioxygenation," in which the carbon bonded to the heteroatoms and the adjacent carbon in the aromatic ring are oxidized. Angular dioxygenation of dibenzofuran, carbazole, dibenzo-*p*-dioxin, and diphenyl ether produces chemically unstable hemiacetal-like intermediates, which are spontaneously converted to 2,2',3-trihydroxybiphenyl, 2'-amino-2,3-dihydroxybiphenyl, 2,2',3-trihydroxydiphenyl ether, and phenol plus catechol, respectively. Angular dioxygenation thus results in the cleavage of the diphenyl ether three-ring structure. The 2'-substituted 2,3-dihydroxybiphenyls that are formed are degraded further by *meta*-cleavage and hydrolysis, resulting in the formation of salicylate (from dibenzofuran; Fortnagel et al., 1990; Strubel et al., 1991), catechol (from dibenzo-*p*-dioxin; Wittich et al., 1992), or anthranilate (from carbazole; Sato et al., 1997), respectively. So far, only a few bacterial strains capable of catalyzing angular dioxygenation have been described, predominantly strains of *Pseudomonas*, *Sphingomonas* and *Terrabacter* species. The first angular dioxygenase described originated from *Sphingomonas wittichii* strain RW1 (Armengaud et al., 1998). Like dioxygenase  $\alpha$ -subunits of angular dioxygenases from other strains of *Sphingomonas*, *Terrabacter* and *Rhodococcus* species, the  $\alpha$ -subunit of the RW1-derived dioxygenase is closely related to the toluene/biphenyl and naphthalene families of Rieske nonheme iron oxygenases (Shepherd and

Lloyd-Jones, 1998; Kasuga et al., 2001; Iida et al., 2002).

These angular dioxygenases do not comprise a monophylogenetic group, but are derived from at least four lineages. None of these genes has been reported thus far in *Pseudomonas* species, even though some angular dioxygenase genes were reported to be located on mobile elements (Iida et al., 2002). In contrast, carbazole dioxygenases of the phthalate family are frequently observed in *Pseudomonas* species and the gene products have been observed to transform dibenzofuran and dibenzo-*p*-dioxin (Sato et al., 1997; Habe et al., 2002), as well as chlorinated derivatives (Habe et al., 2001; Habe et al., 2002).

**ANAEROBIC DEGRADATION OF HALOAROMATIC COMPOUNDS** The capability for denitrification under anaerobic conditions is widespread among *Pseudomonas* species. Additionally, various dehalogenating enzymes do not require oxygen and function under anaerobic conditions. The observation of *Pseudomonas* species degrading chloroaromatics under anaerobic conditions would not be surprising, and the establishment of the *Pseudomonas* sp. strain CBS3 4-chlorobenzoate dehalogenase genes in *Thauera aromatica* T1, which is capable of degrading toluene and 4-hydroxybenzoate under denitrifying conditions, resulted in a derivative mineralizing 4-chlorobenzoate anaerobically (Coschigano et al., 1994).

Under denitrifying conditions, 2-fluoro- and 3-fluoro- as well as 3-chloro- and 4-chlorobenzoate can be degraded readily (Hägglom et al., 1996; Vargas et al., 2000). Analyses have shown, however, that the ability to degrade such halobenzoates under anoxic conditions is widespread among the Proteobacteria, and various strains of *Pseudomonas* species, most closely related to *P. stutzeri* (Song et al., 2000; Vargas et al., 2000), have been isolated that are capable of degrading 2-fluoro-, and 4-fluoro- as well as 3-chlorobenzoate. Evidently, *P. stutzeri* is important for halobenzoate degradation under natural conditions. However, the mechanisms of halobenzoate degradation under denitrifying conditions have not been elucidated.

**IMPORTANCE OF *PSEUDOMONAS* FOR CHLOROAROMATIC DEGRADATION IN SITU** The frequent isolation of strains of *Pseudomonas* species capable of degrading chloroaromatics seems to indicate that they are of some importance for degradation under natural environmental conditions. However, various catabolic pathways for chloroaromatics are plasmid encoded, and gene transfer is known to occur under environmental

conditions. Different plasmids and mobile elements, specifically those involved in the degradation of chlorobenzoate, 2,4-dichlorophenoxyacetic acid (2,4-D) or chloroaniline, have been examined for their transfer under natural conditions, and transconjugants receiving the plasmids have been characterized. Generally, the type of species recovered might be inherent to the environment that provided the selection pressure. Selection pressures might include to the ability to survive in biofilms or to scavenge nutrients. In a detailed study on two surface horizons from an agricultural soil inoculated with a *P. putida* harboring one of two distinct catabolic plasmids, pJP4 (Don and Pemberton, 1981) or pEMT1 (Top et al., 1995), changes were observed in the overall community because of proliferation of the transconjugants (Dejonghe et al., 2000), and a detailed phylogenetic analysis of these transconjugants showed that the plasmids were preferentially acquired and expressed in soil by representatives of *Ralstonia* and *Burkholderia*. Similarly, no *Pseudomonas* species could be isolated after bioaugmentation of a sandy loam soil with pJP4 donor strains (Newby et al., 2000).

Other studies have examined collections of strains obtained from environmental samples (McGowan et al., 1998). Degradation of 2,4-D among easily cultivated organisms is more widespread among strains of *Ralstonia* and *Burkholderia* species than among *Pseudomonas* species. However, such studies are dependent upon the environment analyzed. Subsurface aquifers constitute environments that are physically, chemically and biologically different from surface soils with reduced concentrations and availabilities of oxygen, carbon and nutrients, as well as lower bacterial densities (Ghiorse and Wilson, 1988). The continuous exposure of such aquifers to chlorinated phenoxyacetic acid herbicides has been shown to result in changes in the community composition with a resulting increased abundance of *Pseudomonas* species (De Liphay et al., 2003). Though only one of 50 *Pseudomonas* species mineralized 2,4-D, an important role of *Pseudomonas* on 2,4-D metabolism in situ was suggested. Rhizosphere bacteria, such as fluorescent *Pseudomonas* species, are ecologically adapted to colonize and compete in the rhizosphere environment and it can be speculated that *Pseudomonas* constitute a significant fraction of rhizosphere-associated 2,4-D degraders.

Although *Pseudomonas* sp. strain B13 was isolated as a host of the *clc* element, in inoculation experiments into natural ecosystems, the *clc* element, like *tfd* genes, ended up in *Ralstonia* or related Betaproteobacteria, like *Comamonas* (Zhou and Tiedje, 1995; Ravatn et al., 1998; Springael et al., 2002). This was assumed to

indicate that the *clc* genes are most efficiently expressed in strains belonging to those genera rather than in fluorescent pseudomonads. However, additional factors govern the acquisition and spread of a 3-chlorobenzoate-degrading phenotype in nature. 3-Chlorobenzoate is activated by a chromosomally encoded benzoate dioxygenase and dehydrogenase by organisms harboring chlorocatechol genes. Thus, properties of the benzoate dioxygenase system of possible recipient strains can be regarded as selectivity factors (Bott and Kaplan, 2002). *Pseudomonas* species were prevalent among 3-chlorobenzoate-degrading isolates harboring a chlorocatechol *ortho*-cleavage pathway (Peel and Wyndham, 1999; Krooneman et al., 2000).

Diverse pathways capable of achieving mineralization add another layer of complexity to the degradation of chlorobenzoates. Degradation of chlorobenzoates can occur via chlorocatechol (*clc* pathway), hydrolytic dehalogenation of 4-chlorobenzoate to give 4-hydroxybenzoate (*fc* pathway; Klages and Lingens, 1980), dioxygenation of 3-chlorobenzoate to give 5-chloroprotocatechuate (Nakatsu and Wyndham, 1993), and probably via a fourth pathway with gentisate as intermediate (*gp*-pathway; Krooneman et al., 1996; Krooneman et al., 1998). The archetype of the gentisate pathway for chlorobenzoate degradation was enriched under oxygen-limiting conditions (Krooneman et al., 1996). Obviously, bacteria using this pathway possessed relatively low growth rates on 3-chlorobenzoate and benzoate, along with relatively high substrate and oxygen affinities for these compounds (Krooneman et al., 2000). This is in contrast to bacteria harboring the *clc* pathway, which seem to be characterized by high maximum specific growth rates on aromatic substrates and relatively high apparent half saturation constants. Thus, presumably, bacteria degrading chlorobenzoate via gentisate or protocatechuate might be better adapted to reduced growth rate conditions (i.e., low oxygen and low substrate concentration). Evidently, the spread of the different pathways throughout different taxa has been observed, and *Pseudomonas* species were observed to harbor *clc* genes, whereas the *gp*-pathway was dominant in *Bordetella* and *Alcaligenes* species (Krooneman et al., 2000). Evidently, such clustering is due to the general genetic and metabolic equipment of the organisms. However, gentisate dioxygenase genes were observed in different *P. aeruginosa* isolates (Hickey et al., 2001) and in the genome of *P. aeruginosa* PAO1 (Stover et al., 2000) but not in the genome of *P. putida* KT2440 (Nelson et al., 2002).

Whereas a degradative pathway of chlorobenzoate via gentisate has not been analyzed in

detail, the pathway via protocatechuate has been elucidated and the crucial chlorobenzoate dioxygenase was analyzed also at the genetic level. The host range of *cbaAB* genes for 3-chlorobenzoate 4,5-dioxygenase has been analyzed both in defined mating experiments as well as by analysis of transconjugants (Fulthorpe and Wyndham, 1991; Nakatsu et al., 1995) formed under environmental conditions. Transconjugants were predominantly Betaproteobacteria, but also some *Pseudomonas* transconjugants were observed. However, those transconjugants predominantly formed 3-chlorocatechol from 3-chlorobenzoate, indicating an active benzoate 1,2-dioxygenase was interfering with 3-chlorobenzoate 4,5-dioxygenase. Moreover, 5-chloroprotocatechuate accumulated, indicating that protocatechuate 3,4-dioxygenase cannot adequately deal with this metabolite. In contrast, mineralization of 3-chlorobenzoate was observed in organisms harboring a protocatechuate 4,5-dioxygenase, an enzyme that can dehalogenate 5-chloroprotocatechuate (Kersten et al., 1982). Thus, the host range of the *cbaAB* genes can be correlated with the distribution of the protocatechuate *meta*-ring-fission pathway (Nakatsu et al., 1995). Hosts of the *fc* genotype were, in contrast, mainly *Pseudomonas* (Peel and Wyndham, 1999), which correlates with the ability of *Pseudomonas* to mineralize 4-hydroxybenzoate.

### Biotransformation

Biotransformation refers to the biocatalytic conversion of a preformed substrate into a product by a single or a few enzymatic steps. Although free or immobilized enzymes may be applied as such, cofactor-dependent reactions are performed preferentially in living cells to allow inexpensive cofactor regeneration. Several cofactor-independent enzymes are applied in industry (lipases, nitrilases, isomerases, etc.) and bulk processes are based on the stable action of such enzymes. In contrast, cofactor-dependent whole cell reactions (oxidations, reductions, etc.) still have not been applied to tap their inherent potential. A key element in terms of the economics of these processes is the susceptibility of the living cell to the substrate and other components of the reaction solution and the resulting chemical produced. With the molecular tools available, it is possible to transfer genetic information from a variety of organisms into a suitable host.

Several species of *Pseudomonas* have been shown to be good host strains for the expression of heterologous genes providing considerable potential for biotechnological exploitation. Furthermore, strains of *Pseudomonas* species are

often tolerant of, or resistant to, noxious agents present in soils, including antibiotics, disinfectants, detergents, heavy metals, and organic solvents. Several species of *Pseudomonas*, in particular *P. putida*, have been shown to be good host strains for the expression of heterologous genes (Mermod et al., 1986; Ramos et al., 1987; Cases and de Lorenzo, 1998). As a result, these bacteria are viewed as potential cell factories in a diverse range of biotechnological applications, including bioremediation of contaminated sites (Dejonghe et al., 2000), quality improvement of fossil fuels, for example by desulfurization (Galan et al., 2000), biocatalysis for the production of fine chemicals (Zeyer et al., 1985; Schmid et al., 2001), the production of bioplastics (Olivera et al., 2001), and as agents of plant growth promotion and plant pest control (Walsh et al., 2001).

Moreover, a limited number of catabolic enzymes that typically mediate chemically difficult-to-achieve reactions as a consequence of their regio- and stereoselectivity, and thus have considerable potential as biocatalysts for the production of fine chemicals, have been characterized. Some of the previously unknown catabolic enzymes revealed by genome analysis may equally have promise for industrial biotransformations, such as those for the production of epoxides, substituted catechols, enantiopure alcohols and amides and heterocyclic compounds (Zeyer et al., 1985; Wubbolts and Timmis, 1990; Faber and Franssen, 1993; Schmid et al., 2001). Additionally, identification of the genes involved in the synthesis of poly-hydroxy-(phenyl)alkanoic acids, which are currently being studied as biodegradable aromatic polymers, and their precursors, will aid the development of novel bioplastics (Gorenflo et al., 2001; Olivera et al., 2001).

**BIOTRANSFORMATIONS BY SOLVENT-TOLERANT PSEUDOMONAS** Owing to their versatility and particularly to their chiral and positional selectivities, enzyme-mediated processes have become increasingly important for the production of fine chemicals. Many such processes, however, require cofactor regeneration, which is the major reason for the use of whole-cells biocatalysis. A number of high-value biocatalytic conversions involve apolar substrates and products, such as aliphatic, aromatic and heterocyclic compounds (Schmid et al., 2001). Such substances are generally water-insoluble and toxic to whole cells. By using multiple-phase media, it is often possible to integrate conversion and downstream processing within a single reactor system. Therefore, solvent-tolerance of the microorganisms carrying out the conversions is essential. Unlike *E. coli* or many other microorganisms, *P. putida*

strains, such as DOT-T1 (Ramos et al., 1994) or S12 (De Bont, 1998), tolerate high concentrations of solvents with low logP values, which, coupled to their metabolic versatility, make these bacteria particularly suited for the production of fine chemicals in nonconventional media. The most critical mechanism for solvent-tolerance in these bacteria is the existence of ATP-driven specific solvent-efflux pumps that extrude the solvent and hinder its accumulation in the cell membranes. Solvent-tolerant strains of *P. putida* harbor specific mechanisms that deal with environmental stresses. They have special solvent efflux pumps, and their outer membrane has specific characteristics, including an unusual fatty acid composition. Presumably, other mechanisms (stress proteins, specific compounds overproduced, etc.) involved in tolerance remain to be elucidated. The combination of these adaptive responses not only allows the organisms to withstand solvents but also places them in a far better position to cope with chemicals that are toxic to other organisms. Many of these toxic chemicals are the desired products of industry, including aldehydes, alcohols, hydroxylated aromatics and epoxides.

## Summary

This review has attempted to present an overview of *Pseudomonas* in light of much of the genotypic analyses that were initiated more than 30 years ago and that have served to provide the groundwork for proposing a taxonomic framework based upon estimations of phylogenetic relationships. Certainly, these data comprise the majority of new taxonomic information obtained since the last two compilations of Pseudomonadaceae and *Pseudomonas* in the last two editions of *The Prokaryotes* and play an important role in revealing the heterogeneity of *Pseudomonas* sensu lato, and in improving and stabilizing the taxonomy of *Pseudomonas* sensu stricto and other pseudomonads. The recognition of the “natural” relationships of these bacteria, in turn, should lead to better appreciation and understanding of the overall biology of *Pseudomonas* spp. As new species of *Pseudomonas* are isolated and described, the correlation of metabolic and genetic characteristics with the phylogeny and taxonomy of these organisms should shed more light on the ecological and biotechnological potential of this complex bacterial genus.

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## *Pseudomonas aeruginosa*

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The Gram-negative bacillus *Pseudomonas aeruginosa* is a remarkably adaptable organism. Endowed with a large repertoire of metabolic pathways, *P. aeruginosa* is able to adapt, survive and persist under a broad range of environmental conditions. Equipped with an equally large repertoire of pathogenic mechanisms, *P. aeruginosa* is capable of infecting eukaryotic organisms ranging from social amoeba to humans. The discussion in this chapter will highlight the metabolic and pathogenic diversity of *P. aeruginosa* and the relationship to human infection.

### Cellular Biology and Basic Physiology

*Pseudomonas aeruginosa* was first isolated from a variety of environmental sources by Schroeter in 1872 (Palleroni, 1984). Taxonomy based on 16S rRNA sequences positions *P. aeruginosa* within the class of Gammaproteobacteria and the family Pseudomonadaceae. Like all members of this family, *P. aeruginosa* is metabolically versatile and utilizes over 80 organic compounds as energy and carbon sources (Palleroni, 1984). The organism utilizes the Entner-Doudoroff pathway instead of glycolysis to oxidize sugars including fructose, galactose, glucose and xylose, is oxidase positive, and can grow at temperatures up to 42°C (Palleroni, 1984). Though *P. aeruginosa* is classified as an obligate aerobe, some strains will grow anaerobically by denitrification. ATP can also be generated anaerobically by fermentation of arginine and pyruvate (Vander Wauven et al., 1984; Eschbach et al., 2004). One important consequence of this metabolic diversity is that *P. aeruginosa* is pervasive throughout the hospital setting and persists in respiratory equipment, sinks, tubs and weak antiseptic solutions. This characteristic undoubtedly contributes to the opportunistic nature of *P. aeruginosa* infections.

The colony morphology, pigmentation, and motility properties of *P. aeruginosa* strains can be quite heterogeneous. The prototypical colony is large and smooth with an elevated center giving it the appearance of a fried egg (Palleroni, 1984). Variants with alterations in colony mor-

phology arise during biofilm growth, following exposure to environmental and antibiotic stresses, and during chronic infections of the human airways (Sheehan et al., 1982; Haussler et al., 1999; Haussler et al., 2003; Deziel et al., 2001; Drenkard and Ausubel, 2002; Boles et al., 2004; Von Gotz et al., 2004). Many colony morphology variants also display phenotypic alterations in antibiotic resistance, motility, virulence gene expression, adherence properties, and biofilm formation. The generation of variants with diverse phenotypic properties may contribute to the persistence and pathogenic success of *P. aeruginosa*.

*P. aeruginosa* colonies are commonly pigmented and in fact the species designation, derived from aeruginous meaning “the color of copper rust,” reflects the characteristic blue-green color imparted on colonies by the phenazine pigment pyocyanin. Pyocyanin is a redox-active compound with multiple cytopathic effects towards mammalian cells and is a product unique to *P. aeruginosa* (Lau et al., 2004). Other common pigments include the fluorescent yellow-green siderophore pyoverdinin and the rusty-brown phenazine pyorubin (Palleroni, 1984). Another phenotype observed on solid medium is mucoidy, resulting from the overproduction of alginate. Alginate is an extracellular polysaccharide composed of polymeric L-guluronic acid and D-mannuronic acid (Evans and Linker, 1973). The mucoid phenotype is most common in isolates from the airways of cystic fibrosis (CF) patients and correlates with the onset of chronic infection. Potential roles for alginate in pathogenesis include inhibition of opsonic and nonopsonic killing, increased resistance to antibiotics, and adherence to epithelial cells (May et al., 1991). Alginate is also a matrix component of biofilms formed in the CF airways. The mucoid phenotype is rapidly lost upon passage of *P. aeruginosa* in the laboratory.

*Pseudomonas aeruginosa* is also versatile with respect to motility and chemotaxis. Three distinct forms of motility, swimming, twitching and swarming, allow for movement in liquid and on solid surfaces. In aqueous environments *P. aeru-*

*ginosa* swims by means of a single polar flagella. Movement on solid surfaces is by means of twitching and swarming, whereas twitching is powered by the sequential extension and retraction of type IV pili, while swarming requires both flagella and type IV pili (Bradley, 1980; Kohler et al., 2000; Merz et al., 2000). Chemotactic responses by swimming and twitching have been demonstrated for a wide range of chemicals. Attractants include virtually all compounds known to support *P. aeruginosa* growth (Parales et al., 2004). The attractants (and repellants) are recognized by 26 methyl-accepting chemotactic receptors. These receptors transmit signals to four clusters of chemotaxis genes to modulate swimming, twitching, and cell surface properties (Parales et al., 2004). In contrast, *Escherichia coli*, with a more limited chemotactic response, has only six chemotaxis receptors and a single cluster of chemotaxis genes. The versatility of the *P. aeruginosa* chemotactic response probably co-evolved with its diverse metabolic pathways to allow for taxis towards and degradation of potential energy sources.

### Pathogenesis

Though typically found in soil and water, *P. aeruginosa* is also an opportunistic pathogen of plants, invertebrates and vertebrates (Rahme et al., 1995). Involvement in human disease was first recognized in 1882 when Gessard isolated *P. aeruginosa* from bandages discolored greenish-blue with pyocyanin (Gessard, 1984). *Pseudomonas aeruginosa* is now recognized as a common source of many community acquired and nosocomial infections. The spectrum of *P. aeruginosa* infections ranges from self-limiting folliculitis to life-threatening bacteremia. The most common infections involve the cornea, skin, urinary tract, and respiratory tract, although infections may occur in essentially all anatomical locations. *Pseudomonas aeruginosa* is a leading cause of ventilator-associated pneumonia and urinary tract infections in the intensive care unit (Torres et al., 1990; Richards et al., 1999). Individuals most susceptible to *P. aeruginosa* infection include those with immunodeficiency or epithelium compromised by CF, severe burns, ulcerations, or mechanical abrasions such as those resulting from catheterization (Richards et al., 1999). Host immunodeficiency, combined with a high incidence of antibiotic resistant strains, makes treatment of *P. aeruginosa* infections a serious medical challenge.

*Pseudomonas aeruginosa* infections can be classified as either acute or chronic. Acute infections, such as ventilator-associated pneumonia, are invasive, cytotoxic, and frequently result in systemic infection, septic shock, and mortality

(Parillo et al., 1990; Crouch Brewer et al., 1996). In contrast, the chronic respiratory infections associated with CF, despite heavy colonization of the sputum ( $>10^8$  colony-forming units [cfu]/g), are minimally invasive, noncytotoxic, and rarely progress to systemic infection (Hoiby et al., 2001; Rajan and Saiman, 2002). These chronic infections may persist for decades and ultimately result in lung deterioration and mortality. The link between the underlying genetic defect in CF and increased susceptibility to *P. aeruginosa* infection is unclear. Specific adaptations by *P. aeruginosa* to the CF airways clearly play an important role in the development of persistence. These adaptations include adoption of a biofilm lifestyle, conversion to mucoidy, and the loss of virulence gene expression including type IV pili, flagella, exotoxins, lipopolysaccharide O-antigen, and the type III secretion system (Mahenthiralingam et al., 1994; Dacheux et al., 2001a; Feltman et al., 2001; Boles et al., 2004).

**VIRULENCE TRAITS** The pathogenesis of *P. aeruginosa* is often described as multifactorial since virulence cannot be attributed to a single determinant. Factors with known roles in pathogenesis include adhesins, exotoxins, proteases, hemolysins, and a type III secretion system. Despite this formidable collection of virulence factors, *P. aeruginosa* rarely infects the immunocompetent or in the absence of tissue damage. This seemingly paradoxical finding reflects the poor capacity of *P. aeruginosa* to colonize and breach intact epithelia and explains why loss of epithelial integrity, especially in combination with declined immune function, is usually a prerequisite for *P. aeruginosa* infection. Once entry is gained, however, *P. aeruginosa* infections can progress rapidly. The following discussion will focus on virulence determinants with known roles in pathogenesis.

**ADHERENCE.** Colonization of host tissues by *P. aeruginosa* involves both fimbrial and non-fimbrial adhesins. The best characterized of the fimbrial adhesins is the type IV pili. Composed of a helical polymer of the PilA subunit, the type IV pili localize to the cell poles and account for most of the adhesive properties of *P. aeruginosa* (Hahn, 1997). Type IV pili are thought to mediate adherence to a variety of cultured epithelial cells by binding to host cell glycoproteins and low molecular weight polypeptides (Hahn, 1997). Specific binding to cultured eukaryotic cells expressing asialoganglioside GM1 has been demonstrated for some *P. aeruginosa* strains (Saiman and Prince, 1993). *Pseudomonas aeruginosa* also has three sets of type I fimbriae assembled by the chaperone-usher pathway (Vallet et al., 2001). Fimbriae, encoded by the *cupA* gene cluster, play a role in

adherence to abiotic surfaces and in biofilm formation (Vallet et al., 2001; Vallet et al., 2004). Nonfimbrial adhesins, including lipopolysaccharide, flagella, outer membrane proteins and alginate, also contribute to *P. aeruginosa* adherence.

**EXOTOXIN A.** Produced by most clinical isolates, exotoxin A is the most potent of the *P. aeruginosa* exotoxins with a 50% lethal dose (LD<sub>50</sub>) of 2.5 µg/kg for mice (Liu, 1966; Wick et al., 1990). Its mechanism of action is identical to that of diphtheria toxin; exotoxin A inhibits protein synthesis in eukaryotes by ADP-ribosylating elongation factor 2 (Iglewski and Kabat, 1975). Toxin-deficient mutants are attenuated for virulence in most infection models. The toxin is encoded as a single polypeptide consisting of receptor binding, membrane translocation, and ADP-ribosyltransferase domains (Allured et al., 1997; Wedekind et al., 2001). Intoxication of eukaryotic cells is complex and involves binding of the toxin to its receptor  $\alpha_2$ -macroglobulin, internalization into the endosome, proteolytic cleavage and disulfide bond reduction, transport to the endoplasmic reticulum, and ultimately, translocation of the ADP-ribosyltransferase domain to the cytosol (Ogata et al., 1990; Kounnas et al., 1992). Expression of exotoxin A is induced under the iron-limiting conditions encountered within the host (Hamood et al., 2004).

**PROTEASES.** Much of the tissue damage associated with *P. aeruginosa* infections can be attributed to the zinc-dependent metalloendopeptidases LasB (elastase), LasA, and alkaline protease. Each of these proteases has been shown to contribute to virulence in animal infection models (Nicas and Iglewski, 1985). Although the name implies specificity for elastin, elastase degrades a broad range of substrates including laminin, fibrinogen, collagen, transferrin, complement components, and immunoglobulins (Engel, 2003). Given the role of these proteins in host defense, it is not surprising that mutants lacking LasB cause less tissue damage and are less virulent in experimental infection models. The LasA protease is active against a narrow range of substrates and potentiates the action of LasB, human elastase, and other host proteases by nicking substrates (Engel, 2003). LasB and LasA are both synthesized as proenzymes and are exported across the outer membrane by the general secretory or Xcp pathway (Thanassi and Hultgren, 2000). Similar to LasB, alkaline protease demonstrates broad specificity with preferred substrates being complement components, fibrin, fibrinogen, laminin, and interferon gamma (Moriyama and Homma, 1985; Shibuya et al., 1991). Unlike elastase, alkaline protease lacks elastolytic activity and is

secreted by a type I secretion pathway (Guzzo et al., 1991).

**PHOSPHOLIPASES.** *Pseudomonas aeruginosa* makes two distinct phospholipases, the hemolytic PlcH and the nonhemolytic PlcN, both of which are regulated at the transcriptional level by inorganic phosphate (Shibuya et al., 1991). PlcH and PlcN are 40% identical and share a common substrate, phosphatidylcholine, found in eukaryotic membranes and lung surfactant (Ostroff et al., 1990). PlcH also hydrolyzes sphingomyelin, while PlcN shows specificity for phosphatidylserine. PlcH is cytotoxic and inhibits the respiratory burst in neutrophils (Terada et al., 1999). Mutants lacking PlcH and PlcN are less injurious and impaired in dissemination in burn wound and pneumonia infection models (Ostroff et al., 1989; Wiener-Kronish et al., 1993; Rahme et al., 1995). Many *P. aeruginosa* exoproteins, including PlcH and PlcN, are exported across the outer membrane by the general secretory (Xcp) pathway (Lazdunski et al., 1990). Although Xcp substrates are usually translocated across the inner membrane in a Sec-dependent manner, PlcH and PlcN are translocated across the inner membrane by the twin-arginine translocation system (Voulhoux et al., 2001).

**TYPE III SECRETION.** Type III secretion systems are common to many Gram-negative pathogens and function in the translocation of toxins from adherent bacteria directly into eukaryotic cells. Although *P. aeruginosa* pathogenesis is multifactorial, the type III system is a major determinant of virulence. In tissue culture and vertebrate and invertebrate infection models, type III secretion mutants are significantly attenuated in cytotoxicity and virulence (Nicas and Iglewski, 1984; Sawa et al., 1999; Holder et al., 2001). In human respiratory infections, expression of the type III system is associated with a sixfold increase in the relative risk of mortality (Roy-Burman et al., 2001). The type III system may also play a role in the survival of *P. aeruginosa* in soil and water (Pukatski et al., 2002).

The type III system is used to deliver four toxins, ExoS, ExoT, ExoU, and ExoY, to eukaryotic cells (Kulich et al., 1994; Yahr et al., 1996a; Yahr et al., 1998; Finck-Barbancon et al., 1997). The toxins act in concert to inhibit phagocytosis, promote tissue destruction, and impair wound healing (Finck-Barbancon et al., 1997; Geiser et al., 2001). ExoS and ExoT are related bifunctional toxins with amino-terminal GTPase activating (GAP) and carboxy-terminal ADP-ribosyltransferase domains (Barbieri, 2000). The GAP activity stimulates the Rho, Rac and Cdc42 family of signaling GTPases resulting in rearrangement of the actin cytoskeleton and inhibi-

tion of phagocytosis (Goerhing et al., 1999). Although the GAP activities of ExoS and ExoT are virtually indistinguishable, the toxins have distinct targets for ADP-ribosylation. ExoS ADP-ribosylates members of the Ras family of heterotrimeric GTPases result in cytotoxicity, whereas ExoT ADP-ribosylates Crk proteins leading to disruption of focal adhesions and inhibition of phagocytic activity (Sun and Barbieri, 2003). The biological activities of ExoU and ExoY are not as well defined. ExoU has a patatin-like phospholipase domain with broad activity towards phospholipids and neutral lipids (Sato and Frank, 2004). The phospholipase activity of ExoU irreversibly damages cellular membranes leading to rapid necrosis in cultured mammalian cells (Phillips et al., 2003; Sato et al., 2003). ExoY is homologous to the adenyl cyclase domains of anthrax toxin and CyaA of *Bordetella pertussis* (Yahr et al., 1996a). Translocation of ExoY to eukaryotic cells leads to elevated levels of intracellular cAMP and rearrangement of the actin cytoskeleton. Interestingly, all four toxins require eukaryotic cofactors for maximal catalytic activity (Coburn et al., 1991; Yahr et al., 1998; Sato et al., 2003).

Three additional proteins secreted by the type III system (PcrV, PopB and PopD) play a role in the translocation of toxins to host cells (Vallis et al., 1999). Ring-like oligomeric structures consisting of PopB/D have been visualized by electron microscopy, and when inserted into membranes, these structures are hemolytic (Dacheux et al., 2001b; Schoehn et al., 2003). The type III toxins are thought to be translocated to host cells through a channel formed by the PopB/D oligomer. The third translocation protein PcrV is required for the membrane insertion of PopD (Goure et al., 2004). Neutralizing antibodies directed against PcrV interfere with the translocation reaction and improve survival in pneumonia and burn-wound infection models (Sawa et al., 1999). Active and passive immunization strategies using PcrV are being evaluated for the treatment of human infection.

The type III secretion system (TTSS) is expressed in response to a variety of environmental signals including low  $\text{Ca}^{+2}$  concentrations, serum, and contact with eukaryotic cell surfaces (Iglewski et al., 1975; Vallis et al., 1999). Induction by the low  $\text{Ca}^{+2}$  signal is understood best and is mediated by two distinct signaling mechanisms. Both require ExsA, a positive transcriptional activator of type III gene expression (Frank et al., 1994). The first signaling mechanism involves a membrane-associated adenylate cyclase (CyaB) that catalyzes the formation of cyclic AMP under low  $\text{Ca}^{+2}$  conditions (Wolfgang et al., 2003). The rise in intracellular cAMP activates the cAMP-dependent transcriptional

factor Vfr resulting in transcription of genes encoding the type III secretion. The second pathway coupling low  $\text{Ca}^{+2}$  to TTSS gene expression is mediated directly through the type III secretion channel. Under high  $\text{Ca}^{+2}$  conditions, the secretion channel is inactive and transcription of the TTSS is repressed (Yahr et al., 1996b; McCaw et al., 2002). Transcriptional repression under these conditions is mediated by ExsD, which functions as an anti-activator by binding to and inhibiting ExsA transcriptional activity (McCaw et al., 2002). Under low  $\text{Ca}^{+2}$  conditions, the secretion channel becomes activated and, as a consequence, transcription is derepressed. Transcriptional activation requires ExsC, which functions as an anti-anti-activator by binding to and inhibiting ExsD (Dasgupta et al., 2004). The mechanism whereby ExsC is made available for binding to ExsD under low  $\text{Ca}^{+2}$  conditions is unknown.

**QUORUM SENSING.** An analysis of the *P. aeruginosa* genome reveals that nearly 10% of the open reading frames identified encode regulatory functions (Stover et al., 2000). One of the better-characterized regulatory systems is the cell density-dependent, quorum sensing regulatory network that uses acyl-homoserine lactone (acyl-HSL) signals. *Pseudomonas aeruginosa* makes use of two distinct quorum sensing systems, *las* and *rhl*, to coordinate gene expression with bacterial cell density. Each system consists of an acyl-HSL synthase (LasI and RhlI; Passador et al., 1993), the respective acyl-HSL signals (*N*-[3-oxodecanoyl] homoserine lactone and *N*-butyryl homoserine; Pearson et al., 1994; Pearson et al., 1995), and the corresponding DNA binding regulatory proteins (LasR and RhlR; Gambello et al., 1993). The substrates for signal synthesis by LasI and RhlI are the common cellular metabolites, *S*-adenosyl methionine and acylated acyl carrier protein (Parsek et al., 1999). LasR and RhlR are DNA-binding regulatory proteins that harbor a C-terminal helix-turn-helix DNA binding domain. In addition to LasR and RhlR, a third response regulator has been identified, QscR (Chugani et al., 2001). QscR does not have an associated signal synthase protein, and the signal to which it responds is unclear. These regulators are thought to bind to regions of dyad symmetry upstream of target promoters, termed a “*las*-” or “*rhl*-box.”

In batch culture, the acyl-HSL signals accumulate as a function of cell density. At a critical cell density (a quorum), the acyl-HSLs reach a threshold concentration at which they bind to the cognate transcriptional regulators. Although most *P. aeruginosa* genes controlled by quorum sensing are activated, some are repressed through an unknown mechanism. Three independent DNA microarray studies identified a

common set of 77 quorum-induced genes (Hentzer et al., 2001; Schuster et al., 2003; Wagner et al., 2003). Included in this set are the genes for LasA and LasB proteases, alkaline protease, and genes required for the biosynthesis of rhamnolipids, hydrogen cyanide, and the phenazine antibiotic, pyocyanin. These studies also highlight the important point that quorum sensing responses are modulated by environmental conditions. While there were 77 core genes conserved between studies, there were numerous quorum sensing-regulated genes unique to each study, probably reflecting the different environmental conditions used to grow *P. aeruginosa*. Array studies have also revealed that there is significant overlap between the quorum-sensing regulon and genes regulated by the stationary phase sigma factor, RpoS (Schuster et al., 2004). The overlap between the quorum sensing and stationary phase regulons suggests that the cell can coordinate gene expression upon both the entry into the stationary phase of growth and high cell densities. This is not surprising since these two conditions are likely to occur together.

Quorum sensing in *P. aeruginosa* is a highly integrated regulatory cascade. The *las* system has been shown to control expression of the *rhl* system, while QscR modulates expression of the *las* system (Pesci et al., 1997; Chugani et al., 2001). Finally, a secreted quinolone signal, 2-heptyl-3-hydroxy-4-quinolone (PQS), is known to affect expression of many quorum sensing-regulated virulence factors (Pesci et al., 1999). The *las* system is required for PQS production and the *rhl* system represses PQS synthesis. In addition to the complex interplay between the *las*, *rhl* and PQS systems, there is evidence that multiple regulatory systems can modulate the quorum-sensing response. For example, the GacA-GacS two-component system, MvfR and Vfr have all been shown to influence quorum sensing (Albus et al., 1997; Reimmann et al., 1997; Cao et al., 2001). The multiplicity of input signals and regulatory factors indicate that quorum sensing responses can be fine-tuned to multiple environmental inputs. Finally, and notably, most of the work discussed in this section has been performed in the laboratory strain, PAO1. An analysis of various clinical isolates has demonstrated that some strains do not possess two-functional, quorum-sensing systems (Cabrol et al., 2003). Therefore, while most *P. aeruginosa* isolates do utilize acyl-HSL-based quorum sensing, the regulatory circuitry can vary extensively even within a single species.

Many of the genes regulated by quorum sensing are known virulence factors. Indeed, quorum-sensing mutants have been shown to be avirulent in three different infection model sys-

tems. In the context of infection, quorum sensing is thought to function by allowing *P. aeruginosa* to build to a critical population density before producing quorum-sensing, regulated virulence factors. By delaying the production of virulence factors that bacteria are thought to avoid an immune response by the host, until enough bacterial cells are present to overwhelm the host. However, with *P. aeruginosa* being an opportunistic pathogen, quorum sensing also probably plays an important function for the organism in the environment.

**BIOFILMS.** Biofilms are commonly defined as surface-associated bacterial communities encased within a polymeric matrix. Biofilms are important in *P. aeruginosa* pathogenesis, since they form on the surfaces of medical devices such as catheters, endotracheal tubes, and contact lenses, and are thought to contribute to chronic infection in the airways of cystic fibrosis (CF) patients (Parsek and Singh, 2003). Compared to their free swimming planktonic counterparts, *P. aeruginosa* growing as biofilms possess increased resistance to antibiotics, complement- and antibody-mediated clearance mechanisms, phagocytosis, and biocides (Parsek and Singh, 2003). This inherent resistance makes biofilm infections difficult to treat and in some instances, such as the respiratory infections associated with CF, impossible to eradicate. The mechanistic basis for increased biofilm resistance to antibiotics is complex and likely reflects a combination of the following: diminished metabolic activity within the biofilm, physiological changes associated with biofilm growth that lend themselves to resistance, and poor penetration of the antibiotics (Costerton et al., 1998). Recent studies have suggested that the elevated production of cyclic glucans in biofilm communities can contribute to the antibiotic resistance by physical sequestration of the antibiotic (Mah et al., 2003).

*Pseudomonas aeruginosa* represents one of the paradigm organisms for the study of pure culture laboratory biofilms. Much work has focused on identifying the molecular determinants that contribute to biofilm formation and function. Complicating these analyses is the fact that biofilm development is sensitive to culturing conditions. Gene-encoded functions that influence biofilm formation under one growth condition may be dispensable under a different set of conditions; however, some generalities can be made. Surface appendages, such as flagella and pili, generally contribute to both attachment to surfaces and the development of "mature" biofilms with distinct architecture. Secreted factors and membrane proteins have also been demonstrated to affect biofilm development, as they variously influence cell surface chemistry. The genetic determinants involved in biofilm devel-

opment should be presented taken with a word of caution. Not only do growth conditions influence the biofilm maturation pattern for any given strain, but significant variation is also observed between different *P. aeruginosa* strains.

*Pseudomonas aeruginosa* biofilm formation is thought to proceed through a number of steps that have been identified through microscopic analysis of the biofilm communities over time. These steps include initial attachment, subsequent growth and—biofilm maturation (characterized by cells encased in an extracellular matrix), followed by detachment during which bacteria actively leave the biofilm. Flagella have been implicated in initial attachment to a surface, although certain growth conditions can circumvent this requirement. Type IV pili have also been shown to be required for initial attachment (O'Toole and Kolter, 1998). Type IV-mediated twitching motility has been shown to be important for biofilm maturation, with mutants defective in twitching motility forming biofilms that are loose and unstructured in appearance (Klausen et al., 2003). Pili-associated adhesins have also been shown to play a role in initial attachment. The *cupA* locus, which shows homology to the fimbrial usher chaperone pathway, has also been shown to be important for initial attachment and subsequent development (Vallet et al., 2001). Another determinant affecting attachment is the putative exopolysaccharide encoded by the *psl* operon in nonmucoid strains (Haussler et al., 2003; Jackson et al., 2004; Matsukawa and Greenberg, 2004). Mutations in this locus have a severe attachment-deficient phenotype. A second putative exopolysaccharide biosynthetic gene cluster, the *pel* locus (Jackson et al., 2004), has been identified as a major attachment determinant in other strains.

Several factors influence biofilm maturation. Production of the surfactant, rhamnolipid, is required for maintaining void spaces and channels in mature biofilms (Davey et al., 2003). Overproduction of secreted polysaccharides has also been implicated in the formation of mature biofilms. Mucoid strains of *P. aeruginosa* (those which overproduce alginate) form biofilms with more biomass and distinctive structure when compared to nonmucoid strains (Hentzer et al., 2001). Mucoid biofilms are also more resistant to the antibiotic tobramycin than comparable nonmucoid biofilms. On the other hand, in laboratory-grown biofilms, nonmucoid strains do not appear to use alginate as a part of the extracellular matrix. Finally, quorum sensing has been shown to control biofilm formation under certain growth conditions (Davies et al., 1998). Quorum-sensing mutants in the *las* system form biofilms that are structurally distinct from the wildtype strain. The quorum-sensing mutant biofilms are

also susceptible to the surfactant sodium dodecyl sulfate. The importance of quorum sensing in biofilm development is also dependent upon culture conditions.

## Summary

Because of its clinical and environmental relevance, *P. aeruginosa* continues to be a focus of intense research. This organism is arguably the paradigm for studying both biofilm communities and acyl-HSL-based quorum sensing in the laboratory. In recent years, the sequenced *P. aeruginosa* genome and the availability of DNA microarray technology have contributed to an explosion in our knowledge regarding the basic biology of *P. aeruginosa*. The momentum *P. aeruginosa* genomics has generated should continue, revealing how the regulation of different processes is coordinated and interconnected. Genomics will also facilitate the study of complex systems as researchers explore the interactions of *P. aeruginosa* with eukaryotic hosts and in environmental contexts. One thing is sure, this remarkably versatile organism will continue to surprise us in the future.

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## Phytopathogenic Pseudomonads and Related Plant-Associated Pseudomonads

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Phytopathogenic pseudomonads are a very diverse group of bacteria with respect to their genetics, ecology, and the kinds of diseases they cause. They currently are grouped into approximately 25 species that could easily be placed into at least three distinct genera. Moreover, there are approximately 50 pathovars in the species *P. syringae*, most of which attack different hosts. There also are pathovars in other species. These pseudomonads are worldwide in distribution and cause diseases on most major groups of higher plants. Some of the world's most serious bacterial diseases are caused by pseudomonads, such as *Pseudomonas solanacearum*. Because of the genetic diversity of the phytopathogenic pseudomonads, disease symptomatology in plants ranges from necrotic lesions, spots, cankers, and twig dieback (blights) to hyperplasias (galls, scabs), tissue maceration (rots), and vascular infections (wilts). Some pseudomonads, for example, *P. aeruginosa* and *P. cepacia*, appear to infect both plants and animals, although more work needs to be done on the comparison of strains from both sources.

Research on the phytopathogenic pseudomonads during the 1980s has been narrowly based with greatest emphasis given to such areas as molecular biology, where support funds have been more plentiful. Consequently, considerable progress has been made on understanding the genetic and physiological bases for such characteristics as virulence and host specificity. The development of DNA probes and immunological techniques to detect strains in nature also has progressed well. On the other hand, pseudomonad taxonomy and nomenclature remain perplexing since systematics has received little attention. This is unfortunate, since the defining of taxonomic relationships provides valuable information for those who are searching for clues concerning bacterial properties that relate to pathogenesis and ecological fitness. Many of the studies on the genetics of phytopathogenic

pseudomonads in the 1980s were based on findings from previous investigations comparing the physiological and pathological differences among taxa.

Upon reviewing our previous chapter on the phytopathogenic pseudomonads in the first edition of *The Prokaryotes* (Schroth et al. 1981), it is evident that there has not been a good transfer of basic information to problem solving during this decade. The diseases in general are not better controlled today than they were ten years ago and not much more is known about the ecology of the causal agents with the exception of a few taxa, such as *P. syringae* pv. *syringae*. There also have not been any particularly new innovations on management of diseases. However, it is expected that with time, the application of new information gained in the 1980s from studying such factors as virulence and host specificity will result in the development of improved approaches to control plant diseases.

In contrast to the phytopathogenic pseudomonads, there has been great activity in studying the ecology, physiology, and genetics of nonpathogenic plant-associated pseudomonads, particularly those that colonize the spermosphere and rhizosphere (Schroth and Becker, 1990; Schroth et al., 1984; Weller, 1988). Greatest interest has been with those pseudomonads that affect plant growth through their interactions with fungal plant pathogens or by their direct effect on the roots, possibly because of hormone production. Some mention is given to these microorganisms in this chapter because they colonize plant parts and in some cases appear to damage the plant. Furthermore, there is no clear demarcation between parasitism and saprophytism.

### An Overview of Systematics

The evidence suggesting that the *Pseudomonas* phytopathogens belong to at least three different genera comes from various sources including rRNA-DNA homologies (Byng et al., 1983; De

Vos et al., 1985; Palleroni et al., 1973), cellular fatty acid composition and the quinone system (Oyaizu and Komagata, 1983), and enzymological patterning (Byng et al., 1983; Whitaker et al., 1981) (see also 160 and 161). The three groups containing plant pathogens are the *P. fluorescens* group (called the “fluorescents” because most form water-soluble pigments that fluoresce in visible and ultraviolet light), the *P. solanacearum*—*P. cepacia* group, and the *P. avenae* group. The latter two have been lumped together in this article as the “nonfluorescents.”

Initial assignment of plant pathogens to the nonfluorescent group is usually based not on absence of fluorescent but upon the accumulation of poly- $\beta$ -hydroxybutyrate (PHB) as a storage reserve material; the nonfluorescents usually accumulate this polymer whereas the fluorescents do not. The reliability of PHB accumulation for distinguishing the nonfluorescents from fluorescents is illustrated by the example of *P. pseudoalcaligenes* subsp. *citruilli*. This phytopathogen accumulates PHB but nevertheless had been classified on the basis of some other properties as a subspecies of *P. pseudoalcaligenes* (Schaad et al., 1978), one of the few nonfluorescent species that belong to the fluorescent group. More recently, however, this pathogen has been reassigned to the *P. avenae* nonfluorescent, PHB-positive group on the basis of rRNA cistron similarities (De Vos et al., 1985). It is likely that *P. pseudoalcaligenes* subsp. *konjaci* also belongs in this group.

The title-role trait of production of water-soluble fluorescent pigments on King’s medium B (King et al., 1954) is less reliable than PHB accumulation for assignment to a group. Although most members of the fluorescent group produce fluorescent pigments, exceptions exist, such as *P. agarici*, *P. corrugata*, *P. syringae* pv. *cannabina*, and *P. s.* pv. *sesami*. Others do not produce fluorescent pigments on King’s medium B but do so on other media; some strains of normally fluorescent pigment-producing pathovars may be nonfluorescent (Hildebrand and Schroth, 1972). Fluorescent pigment production in some instances can be induced by iron-chelating techniques (Garibaldi, 1967) or by use of media other than King’s medium B, such as that described for *P. s.* pv. *persicae* by Luisetti et al. (1972). Certain members of the nonfluorescent groups, such as *P. cepacia* and *P. gladioli*, produce diffusible yellow pigments similar in appearance to fluorescent pigments except that they do not fluoresce. Consequently, pigments must be examined under ultraviolet light (Hildebrand et al., 1973). Other properties such as utilization of cellobiose and D-arabinose can be used to identify many of the nonfluorescent pseudomonads.

## The Fluorescent Group of Phytopathogenic Pseudomonads

The taxonomy of the fluorescent group of phytopathogenic pseudomonads has been debated for years. Many of the original species were named primarily on the basis of the host plant from which the pathogen was isolated and its presumed host range, not on a comparative phenotypic characterization. The problem of inadequate characterization has remained with us. Few species of phytopathogenic pseudomonads were included in the 1980 *Approved Lists of Bacterial Names* (Skerman et al., 1980) because of inadequate characterization. The only names of phytopathogenic species and subspecies of the fluorescent group which appear in the 1980 *Approved Lists* and the 1985 update (Moore et al., 1985) are *P. agarici*, *P. amygdali*, *P. caricapapayae*, *P. cichorii*, *P. coronafaciens*, *P. corrugata*, *P. fuscovaginae*, *P. marginalis*, *P. meliae*, *P. syringae*, *P. syringae* subsp. *savastanoi*, *P. tolaasi*, and *P. viridiflava*. Because of the problem of inadequate characterization, a scheme was developed which designated the many distinct pathogens as pathovars of several recognized species, primarily *P. syringae*. This was adopted as an interim expedient; accordingly, a list of pathovars and related information was published (Dye et al., 1980).

Although the pathovar system was viewed as an interim expedient, and there have been many complaints about it, insufficient work has been done to replace it with an improved, more acceptable taxonomic scheme. In one study (Janse, 1982), a pathovar of *P. syringae* was elevated to the level of subspecies, *P. syringae* subsp. *savastanoi*, but this work was seriously flawed because the properties used to distinguish it from *P. syringae* do not separate it from closely related pathovars. A comprehensive comparative study is critically needed of all recognized species and pathovars.

Considerable information is available indicating that the various pathovars of *P. syringae* are for the most part distinct entities and deserve taxonomic recognition. DNA-DNA homologies within the *P. syringae* group of pathovars (Table 1) range from 37 to 100% (Denny et al., 1988a; Palleroni et al., 1972; Pecknold and Grogan, 1973). Furthermore, the range of homologies within a pathovar is similar to that within *P. aeruginosa*, a so-called “good” species. Usually, strains of a pathovar are phenotypically similar to each other and can be distinguished on the basis of nutritional utilization patterns from strains of other pathovars (Denny, 1988; Hildebrand, 1973; Hildebrand et al., 1988). Table 2 lists some properties useful for identification of certain pathovars.

A system of three-dimensional modeling of various bacterial characteristics has been

Table 1. DNA-DNA homology values for fluorescent phytopathogenic pseudomonads and related species.

Tested strains	Reference strains					
	<i>P. s. syringae</i>	<i>P. s. aptata</i>	<i>P. s. morsprunorum</i>	<i>P. s. phaseolicola</i>	<i>P. s. tomato</i>	<i>P. cichorii</i>
<i>P. s. aptata</i>	85–86	95–100	70–75	54		
<i>P. s. cichorii</i>	32–39	36	32	31–45	36	78–96
<i>P. s. coronafaciens</i>	53	51	46	43		32
<i>P. s. delphinii</i>	64	53	58	55		45
<i>P. fluorescens</i>	21–23	29	25		34	19
<i>P. s. glycinea</i>	55–66	59	82	79–87		36
<i>P. s. helianthi</i>	67	68	57	60		34
<i>P. s. lachrymans</i>	60–66	61–67	78–81	78		
<i>P. marginalis</i>	12–22					18–25
<i>P. s. mori</i> “var <i>huszti</i> ”	60–79		85	83	70	
<i>P. s. morsprunorum</i>	66–70	75–77	98–100	80		
<i>P. s. panaci</i>	82	87	56	60		33
<i>P. s. phaseolicola</i>	60–63	60	83–84	98–100	63	34
<i>P. s. pisi</i>	79	87	59	59		34
<i>P. putida</i>	16–21			19		22
<i>P. s. savastanoi</i>	53–60		82	80	61–66	
<i>P. s. syringae</i>	95–100	86–89	65–68	56–69	66	38–41
<i>P. s. tabaci</i>	71–75	72–74	80–82	78		
<i>P. s. tomato</i>	58–60	56	56–69	54–61		35–41
<i>P. viridiflava</i>	37–41	41	40	38		38–42

After Palleroni et al. (1972) and Pecknold and Grogan (1973).

developed to help show bacterial relationships (Hildebrand et al., 1982; Hildebrand et al., 1984). DNA-DNA hybridization data most often are used to model the relationships; such a model has been made for a limited number of pathovars of *P. syringae* (Hildebrand et al., 1988). A set of coordinates was given to enable the preparation of a physical model of the three-dimensional relationships. These models are useful because phenotypic characteristics can easily be superimposed (mapped) upon them. When this is done, it is observed that strains having a given property usually lie adjacent to each other forming a cluster (Hildebrand et al., 1987). Examination of the boundaries of a phenotypic cluster often indicates why there are problems in the identification of strains; some strains appear to be “intermediate” between clusters. The mapping also indicates the range of variation that might be expected for specific taxa. Pathogenicity also can be mapped upon these models; it behaves as any other phenotypic property. The boundaries of host ranges often overlap in different ways among closely related strains. Examples of this are *P. s. pv. maculicola*, which is phenotypically closely related to *P. s. pv. tomato*. It infects tomato plants and crucifers; *P. s. pv. antirrhini*, which also is phenotypically close to *P. s. pv. tomato*, infects tomato (Hildebrand et al., 1984). It follows, therefore, that pathogenicity, the basis of the pathover system, is not a completely reliable character for the identification of a pathogen (Hildebrand et al., 1987). Rather, identification should be based upon phenotypic characterization and genetic information such

as DNA hybridization or restriction fragment-length polymorphisms (RFLPs). This should be complemented later by pathogenicity and host range tests.

The taxonomic situation in the saprophytic group of fluorescent pseudomonads, which includes many root-associated strains, is similar to that occurring in the pathogens. Stanier et al. (1966) in their nutritional study of the nonphytopathogenic pseudomonads, indicated that they took a broad view in proposing species. They placed most strains into two species, *P. fluorescens* and *P. putida*, which then were divided into several biotypes or biovars. Later, however, Micheal Doudoroff told us that he believed that these two species represented at least 500 actual species.

Reasons for the existing confusion are evident by examination of the DNA-DNA hybridization study of Palleroni et al. (1972). They showed that the homology of 15 strains of *P. putida* to the type strain ranged from 18 to 86%, a very large range to represent a species. This strain also had much greater homology to a *P. fluorescens* strain (45%) than to a number of *P. putida* strains. Likewise, the range of homology values within biovars 1, 2, and 3 of *P. fluorescens* was very large (21 to 81%). Most values were from about 50 to 65%. The relatively low genetic relationships among the strains studied thus far suggests that phenotypic variability also should be great. This is the case; many of the tests presented in *Bergey's Manual of Systematic Bacteriology* (Palleroni, 1984b) are listed as being variable for various taxa (see also Introduction to the Family Pseudomonadaceae in the second edition).

Table 2. Diagnostic tests for identification of *Pseudomonas viridiflava* and some *Pseudomonas syringae* pathovars.

	Levan formed	Pectate gel <sup>a</sup>	Rutin glycosidase	β-Glucosidase	Adonitol	Anthranilate	Betaine	Erythritol	D,L-Homoserine	Inositol	L-Lactate	Mannitol	Quinate	Sorbitol	D-Tartrate	L-Tartrate	Trigonelline
<i>P. viridiflava</i>	-	4.6-8.5	+	+	-	-	+	+	-	+	+	+	+	+	+	-	+
<i>P. s. syringae</i>	+	-	+	+	-	-	+	+	-	+	+	+	+	+	few +	-	+
<i>P. s. antirrhini</i>	+	4.6	+	+	-	-	+	-	+	+	-	+	+	+	+	-	+
<i>P. s. aptata</i>	+	-	+	+	-	-	+	+	-	+	+	+	+	+	slow	-	+
<i>P. s. atrofaciens</i>	+	NT	+	V	-	-	+	+	-	+	+	+	+	+	-	-	+
<i>P. s. cannabina</i>	W	4.6	NT	-	-	-	+	-	-	-	-	-	-	-	-	-	-
<i>P. s. coronafaciens</i>	+	-	+	+	-	-	+	+	-	+	-	+	+	+	-	-	-
<i>P. s. delphinii</i>	-	4.6	+	+	-	-	+	+	-	+	-	+	+	+	-	-	+
<i>P. s. criobotryae</i>	+	4.6	+	+	-	-	+	+	-	-	-	-	+	+	-	+	+
<i>P. s. garcae</i>	+	-	NT	+	-	-	+	+	-	+	-	+	+	+	-	-	-
<i>P. s. glycinea</i>	+	4.6	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+
<i>P. s. lachrymans</i>	+	4.6-8.5	+	+	-	-	-	+	-	+	-	+	+	+	-	+	+
<i>P. s. mori</i>	+	4.6	+	-	few +	-	V	-	-	+	-	+	-	V	-	-	+
<i>P. s. mori</i> "var. <i>huszi</i> "	+	4.6	+	-	+	-	+	-	-	+	-	+	-	+	-	-	+
<i>P. s. morsprunorum</i>	+	4.6	+	-	-	+	+	slow	-	+	-	+	+	+	-	+	+
<i>P. s. papulans</i>	-	NT	+	+	-	-	+	+	-	+	+	+	+	+	-	-	-
<i>P. s. passifloriae</i>	-	NT	+	+	-	-	+	+	-	+	-	+	+	+	-	-	-
<i>P. s. persicae</i>	+	4.6	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-
<i>P. s. phaseolicola</i>	+	4.6	+	-	-	-	V	-	-	-	-	-	+	-	-	-	+
<i>P. s. pisi</i>	+	-	+	V	-	-	+	V	+	+	V	+	+	+	-	-	+
<i>P. s. ribicola</i>	-	NT	+	-	-	-	slow	-	-	+	-	+	+	+	-	-	+
<i>p. s. savastanoi</i>	-	4.6	+	-	-	V	+	-	-	V	-	+	-	+	-	V	V
<i>P. s. sesami</i>	+	4.6-8.5	+	-	-	-	V	+	-	+	-	-	+	-	-	+	+
<i>P. s. striafaciens</i>	+	NT	+	+	-	-	+	-	-	+	-	+	-	+	-	-	-
<i>P. s. tabaci</i>	+	4.6-8.5	+	+	-	-	+	+	-	+	-	+	+	+	-	+	+
<i>P. s. tomato</i>	+	4.6	+	+	-	-	+	-	-	+	-	+	+	+	+	-	+

Symbols: +, product formed or substrate utilized; -, product not formed or substrate not utilized; V, variable results with different strains; W, weak positive.

<sup>a</sup>Indicates the pH at which pitting (sinking of colonies) occurs on pectate gels; -, no action on pectate; NT, not tested. For best results, the low pH pectate gels should be at pH 4.6 or lower (Burki, 1973).

From Hildebrand and Schroth (1972), Hildebrand and Caesar (1989), Hildebrand et al. (1988), and D. C. Hildebrand (unpublished observations).

Identification of root-associated pseudomonads usually is difficult. Many strains appear to be different from those on which various taxonomic keys have been based. Their properties appear to be "intermediate" among several recognized species or biovars. In many cases, they cannot be assigned to either *P. putida* or *P. fluorescens* with certainty. As with the phytopathogens, a very large comprehensive DNA hybridization study is needed to provide background for phenotypic mapping. The completion of this should enable an understanding of the relationships among strains and the development of a more useful identification scheme.

### The Nonfluorescent Group of Phytopathogenic *Pseudomonads*

The nonfluorescent phytopathogenic pseudomonads belong to two different rRNA branches (De Vos et al., 1985). Previous interpretations of rRNA-DNA homology data (Byng et al., 1983;

Kerstens and De Ley, 1984) have suggested that *Alcaligenes* species are intermingled among various pseudomonads on these branches. Three-dimensional modeling of rRNA-DNA homology data, however, suggests quite the contrary—*Alcaligenes* strains form their own cluster that are distinct from either pseudomonad branch (D. Hildebrand, unpublished observations).

The *P. acidovorans* branch consists of the recognized phytopathogenic species *P. asplenii*, *P. avenae*, *P. pseudoalcaligenes* subsp. *citrulli*, and *P. rubrilineans*, and as noted earlier, probably *P. pseudoalcaligenes* subsp. *konjaci*. The members of this group have not been studied well and their overall relationship to each other and other members of the *P. acidovorans* (now *Comamonas acidovorans*) group are unknown.

*P. solanacearum* and related species comprise the second nonfluorescent rRNA branch. The recognized phytopathogenic species in this group include *P. andropogonis*, *P. caryophyllii*, *P. cepacia*, *P. gladioli*, *P. glumae*, *P. rubrisubalbi-*

*cans*, and *P. woodsii*. Unlike the fluorescent group, the species concept in this group of phytopathogenic pseudomonads always has been broad. *P. solanacearum* in particular has been extensively studied since its description by Smith (1896). Many of these studies—including DNA-DNA homology (range: 54–100%), physiological tests, and pathogenicity tests—indicated that this species consisted of a heterogeneous group of organisms. Just as there is little agreement about the subdivision into species of the *P. syringae* group of fluorescent pseudomonads, there is little agreement about the subdivision of *P. solanacearum* into groups. Okabe and Goto (1963) reported that *P. solanacearum* strains could be divided into 40 or more groups by biochemical and serological properties, and phage and bacteriocin reactions. These groups did not correlate in general with the 13 pathovars they designated (Okabe and Goto, 1961). Hayward (1964) indicated that *P. solanacearum* strains fell into four biovars on the basis of phenotypic characteristics, whereas Baptist et al. (1971) discerned only two by enzyme electrophoresis studies. Most workers generally use either the three races scheme described by Buddenhagen and Kelman (1964) or the four biovars system described by Hayward (1964). It is recognized, however, that these races (Buddenhagen and Kelman, 1964) or biovars (Harris, 1972; Hayward, 1964; Keshwal and Joshi, 1976) are not uniform, but consist of numerous and unknown pathovars or subtypes.

Recent work with RFLPs (Cook and Sequeira, 1988; Cook et al., 1989) suggests that some of these groups could be redefined. Their conclusions were that *P. solanacearum* could be divided into two distinct groups. Group 1 included strains of race 1 biovars 3 and 4; group 2 included strains of race 1 biovar 1 and races 2 and 3. Furthermore, they could not distinguish strains of the pathogen by both race and biovar simultaneously. Race 1 was highly variable strains of race 2 (from banana) fell into 3 distinct groups, and race 3 strains were homogeneous. Whereas these results are encouraging, a word of caution must be made about taxonomic conclusions drawn through use of RFLPs. RFLPs may be helpful for the identification of various groups of strains. However, the relationships of groups to each other is much more difficult to ascertain using RFLPs. We have found (unpublished observations) that relationships derived from RFLP data often do not correlate with relationships obtained from DNA hybridization data. For example, RFLP data suggest that *X. campestris* pv. *phaseoli* “var. *fuscans*” is quite distinct from *X. campestris* pv. *phaseoli* (Gabriel et al., 1989). Our DNA hybridization work confirms this relationship. On the other hand, the RFLP

data indicated that *X. c.* pv. *phaseoli* was as distantly related to *X. c.* pv. *glycines* as to *X. c.* pv. *campestris*. DNA hybridization indicates that *X. c.* pv. *phaseoli* is closely related to *X. c.* pv. *glycines* but distantly related to *X. c.* pv. *campestris*.

*P. cepacia* represents another large and presumably diverse group of phytopathogens; the DNA-DNA homology ranges from 46 to 79% (Ballard et al., 1970). It was originally described as a pathogen causing soft-rot disease of onions, but later was broadened to include strains isolated from soil, water, and clinical specimens previously known as *P. multivorans* and *P. kingii* (Ballard et al., 1970; Snell et al., 1972; Sinsabaugh and Howard, 1975). *P. cepacia* is the most nutritionally versatile of all of the pseudomonads, pathogenic or saprophytic (Ballard et al., 1970); fourteen of the 18 strains tested utilized at least 102 of the 136 substrates screened.

*P. gladioli* appears to be a somewhat more tightly clustered species than either *P. solanacearum* or *P. cepacia*. Originally described as causing a rot of gladiolus, its description was broadened by Ballard et al. (1970) to include *P. allicola*, another onion pathogen. DNA-DNA homologies (79–82%) suggested a close relationship between these bacteria but, if the situation in the *P. syringae* group of pathogens is any indication, these organisms probably have distinctive pathogenic properties and should be taxonomically separated. Several phenotypic differences also were observed, such as the propensity of *P. allicola* to produce a darkening of culture media upon aging and a negative or very weak oxidase reaction.

*P. caryophylli*, the carnation pathogen, is another noteworthy plant-pathogenic species. It may be a very homogeneous species since DNA-DNA homologies of three strains ranged from 99 to 100% (Ballard et al., 1970). This species is nutritionally less versatile than either *P. cepacia* or *P. gladioli*, and it is more host- or habitat-restricted.

*P. andropogonis* is an interesting organism. It is placed as an intermediate between the *P. solanacearum* and *P. acidovorans* rRNA branches on the basis of three-dimensional modeling of rRNA-DNA hybridization data. Although it appears more closely related to *P. solanacearum* on the basis of rRNA similarity (De Vos et al., 1985), it belongs in an enzymological patterning group found otherwise only in the *P. acidovorans* rRNA branch (Byng et al., 1983).

## Genetics and Mechanisms of Pathogenesis

Major developments in the genetics of phytopathogenic pseudomonads occurred during the



Table 3. Some genetic studies in phytopathogenic pseudomonads.<sup>a</sup>

Study	Species tested <sup>b</sup>														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Conjugation	+			+	+	+	+	+	+	+	+	+	+	+	+
Transformation	+			+	+	+	+								
Transduction				+											
Libraries	+	+	+	+	+	+	+	+	+	+	+				
Tn mutagenesis	+	+		+	+	+	+	+	+	+					+
IS elements			+		*		+								
Native plasmids	+				+	+	+	+	+	+	+	+	+	+	+
Chromosome mapping	+					+		+				+			
MEX mutants	+			+		+	+	+	+	+					
RELP analysis	+		+		+	+		+	+	+	+	+	+		
Cloned genes:															
<i>avr/hsp</i>	+					+	*			*					
<i>hrp/hri/vir</i>	+			(*)	*	*	*		+	+	+				
<i>path/lem</i>					+	+									
<i>pgl, egl</i>	+	+													
toxin genes					+	+	+	+	+						+
<i>recA</i>			+			+	+								
<i>argF</i>						*									
Cu <sup>r</sup>											*				
<i>flu/sid</i>			+												
<i>ice</i>			*												
<i>iaa</i>				*											
<i>tzs</i>	*			*											

<sup>a</sup>The table is not meant to be inclusive of all group members or relevant genes. Tn: transposon; IS: insertion sequence; MEX: marker exchange; RFLP: restriction fragment length polymorphism. Gene designations: *avr*: avirulence; *hsp*: host specificity; *hrp/hri*: hypersensitive reaction and pathogenicity; *path*: pathogenicity; *lem*: lesion formation; *egl*: endogalacturonate lyase; *pgl*: polygalacturonate lyase; *recA*: homologous recombination; *argF*: ornithine carbamoyltransferase; Cu<sup>r</sup>: copper resistance; *flu/sid*: fluorescence/siderophore genes; *ice*: ice nucleation gene; *iaa*: indoleacetic acid production; *tzs*: transzeatin synthesis; (\*): function established in a related pathovar.

<sup>b</sup>Symbols: A: *P. solanacearum*; B: *P. viridiflava*; C: *P. cepacia*; D: *P. syringae* (nonpathogenic); E–O: *P. syringae* pathovars; E: *savastanoi*; F: *syringae*; G: *phaseolicola*; H: *glycinea*; I: *tabaci*; J: *tomato*; K: *maculicola*; L: *morsprunorum*; M: *atropurpurea*; N: *mori*; O: *pisi*.

1980s following an exploratory phase during the 1970s. Although the full impact of these developments on physiological, biochemical and other areas has not yet been realized, we seem to be close to making major advances in our understanding of pathogenic mechanisms of these bacteria (Kerr, 1987). Table 3 shows the range of activity and focus in several “model” members. Only some areas, in which more definitive studies have been carried out, are discussed. Several excellent reviews are cited which cover these and many other relevant subjects.

### Genetic Linkage Analysis

Conventional methods for chromosome mapping have been applied to four pathovars of *P. syringae*. The most extensive linkage analysis available is in *P. s. syringae* (see Holloway and Morgan, 1986). The order and spacing of isofunctional loci on this chromosome resemble overall that in the more extensively mapped chromosomes of *P. aeruginosa* and *P. putida*. The relative order of nine auxotrophic loci and an antibiotic

resistance gene has been determined in the *P. s. pv. glycinea* by three factor crosses (Leary and Fullbright, 1982). Several pairs of loci showed transductional linkage in *P. s. pv. morsprunorum* (Errington and Vivian, 1981). Co-transfer of genetic markers was also reported in *P. solanacearum* (Boucher and Sequeira, 1978).

There are no long-range linkage data on genes related to pathogenicity, toxin, or phytohormone production except for those located on plasmids. In *P. solanacearum*, a locus related to methionine biosynthesis (*met*) appears to be linked to a group of pathogenicity genes (*hrp*), a host specificity gene and genes determining acridine orange resistance, lipo- and exopolysaccharide production and composition, and production of a brown pigment in rich medium (Boucher et al., 1987, 1988a, 1988b). Some short-range linkage information has been provided by cloning experiments. For example, several groups or clusters of functionally related genes have been cloned in single cosmids and genomic Tn5 insertion mutants altered in pathogenicity or toxin production. These have been localized in single large

restriction endonuclease fragments. Examples are the *hrp* clusters of *P. syringae* pathovars *phaseolicola*, *glycinea*, and *syringae*, and *P. solanacearum* (in the last two the region may include host specificity genes); genes for phaseolotoxin synthesis along with a gene for the phaseolotoxin-insensitive ornithine carbamoyl-transferase in *P. s. pv. phaseolicola*; genes involved in tabtoxin, syringomycin/syringotoxin production in pathovars *tabaci* and *syringae*, respectively; and the plasmid-borne genes for indole acetic acid (IAA) synthesis and copper resistance in pathovars *savastanoi* and *tomato*, respectively (Barta et al., 1988; Comai and Kosuge, 1980; Comai et al., 1982; Cooksey 1987; Glass and Kosuge, 1986; Huang et al., 1988; Joe et al., 1988; Lindgren et al., 1986; Mellano and Cooksey, 1988; Morgan and Chatterjee, 1985, 1988; Peet et al., 1986; Peet and Panopoulos, 1988; Xu and Gross, 1988a, 1988b; T. V. Huynh, D. Dahlbeck, and B. J. Staskawicz, unpublished observations).

The chromosomes of the well-studied *Pseudomonas* species contain "supraoperonic clusters" that include functionally related genes. reference is not an exact match (Wheels (1975), and Holloway and Morgan (1986) discussed the possible evolutionary significance of this features and underlying mechanisms that may have shaped the evolution of *Pseudomonas* chromosomes. It would be interesting to know if the various clusters of genes involved in pathogenicity, toxin production, or other aspects of *Pseudomonas*-plant interactions are located on a separate chromosomal arc like the catabolic genes in the nonphytopathogenic members.

### Gene Transfer, Cloning, and Transposon Delivery Systems

Several conjugative plasmids have been described in the phytopathogenic *Pseudomonas* (Coplin, 1982; Panopoulos and Peet, 1985; Shaw, 1987). However, these have not been exploited to a great extent in genetic studies. Researchers have instead used other such elements developed for similar work with *Rhizobium*, *P. aeruginosa*, and other nonenteric prokaryotes. These elements are related to or derived from the broad host-range plasmids of the IncP, IncW, and IncQ groups. A wide variety of modifications (size reduction, addition/inactivation/deletion or substitution of selectable markers or mobilization/replication origins, addition of the *cos* fragment from coliphage lambda, incorporation of strong or regulatable promoters, synthetic multi-linkers, transcriptional terminators, easily scorable or assayable markers, and promoterless reporter genes) has produced an extensive array of versatile tools for cloning, transposon mutagenesis, analysis of promoter function, and

other similar investigations (Denhardt and Colasanti 1988; Franklin 1985). Some applications of these tools have been reviewed in Mills (1985), and Chatterjee and Vidaver (1986).

### Plasmids

The presence of one or more plasmids in this group of bacteria is the rule rather than the exception (Coplin, 1982; Panopoulos and Peet, 1985; Shaw, 1987) but the plasmid complement is rarely identical among strains of the same infra-subspecific group (Shaw, 1987). Several associations between plasmids and phytopathologically significant traits have been reported. Examples are the megaplasmids of *P. solanacearum* (Boucher et al., 1986, 1988a, 1988b) and the IAA/cytokinin plasmids of *P. s. pv. savastanoi*, which are discussed elsewhere in this chapter, and plasmids involved in production of the phytotoxin coronatine in several pathovars (Bender et al., 1987; Sato et al., 1983; Willis and Leary, 1984). A plasmid of *P. s. pv. phaseolicola* reversibly integrates into the chromosome (Curiale and Mills, 1982) and a chromosome-mobilizing agent occurs in *P. s. pv. mors-prunorum* (Errington and Vivian, 1981). The copper resistance plasmid of *P. s. pv. tomato* (Cooksey, 1987) and the transmissible streptomycin resistance elements in *P. s. pv. lachrymans* (Yano et al., 1983) and *P. s. pv. papulans* (Burr et al., 1988) are three other examples with agronomic significance.

### Phenotypes of Mutants Altered in Plant Responses

There is general consensus that when a search is undertaken for the isolation of mutants affected in pathogenicity, virulence, or other similar properties, auxotrophic requirements must not be involved because they may prevent pathogen multiplication in the plant even if other mutations are not present. This precaution has been applied in all relevant cases discussed in this chapter.

The search for mutants that remain prototrophic but are altered in pathogenicity on host cultivars or in the reactions they elicit on non-host species has produced four basic mutant types: 1) the "null" type (e.g.,  $Hrp^-$ ,  $Mri^-$  that are unable to elicit hypersensitive responses (HR) on either non-host plants (Klement, 1963) or on susceptible cultivars of the host species 2) those unable to cause disease symptoms on either leaves or other susceptible organs such as pods (e.g.  $Lem^-$ ,  $Path^-$ ) but still able to elicit the HR reaction; 3) those no longer eliciting HR on a resistant host cultivar(s) but produce instead typ-

ical disease symptoms ("compatible" reaction); 4) mutants with reduced host range, having lost the ability to cause disease on some host species cultivars but retaining full virulence toward others and eliciting HR on the formerly susceptible cultivars and on non-host species. Attenuated mutants that show varying degrees of residual pathogenicity (e.g., smaller or fewer lesions, or require higher inoculum concentration to produce symptoms) with either normal or reduced ability to elicit HR on one or more non-host plants at given inoculum concentrations have also been obtained. It is not certain that these mutants represent a genetically distinct class since partial inactivation of genes or generation of promoters by Tn5, the most commonly used transposon, could in theory lead to partial loss of target gene function (Deasey and Matthysse, 1988; Harper et al., 1987; Joe et al., 1988; Lindgren et al., 1986; Malik et al., 1987; Niepold et al., 1985; Salch and Shaw, 1988; Somlyai et al., 1986; Willis et al., 1990). Some of the mutant types above also have been isolated in other phytopathogenic bacteria (Daniels et al., 1988). However, no mutants that are pathogenic but unable to elicit HR on a non-host have been found. Types 1 and 3 above exemplify two distinct categories of genes that are further discussed below.

#### The *hrp* Clusters of *P. syringae* Pathovars and *P. solanacearum*

Studies by Lindgren et al. (1986) showed that a contiguous region (about 20 kb) and an additional unlinked locus in the *P. s. pv. phaseolicola* genome encoded functions that were necessary for the expression of two phenotypes thought to be mutually exclusive, namely the production of primary disease symptoms (watersoaked lesions) on bean, the susceptible host, and the elicitation of the hypersensitive reaction on tobacco and on three additional non-host species (tomato, soybean, and cowpea) that were examined. The acronym *hrp* (phonetic "harp," for hypersensitive reaction and pathogenicity) was used to designate these genes. Although other designations have been used for phenotypically similar mutants (e.g. Hri, Path/HR), the *hrp* acronym has been adopted for such mutants in other pathovars of *P. syringae* (Huang et al., 1988; T. V. Huynh, D. Dahlbeck, and B. J. Staskawicz, unpublished observations) and in *P. solanacearum* (Boucher et al., 1987, 1988a, 1988b) which have *hrp* clusters of similar size. DNA hybridization, marker exchange mutagenesis, or interpathovar complementation showed that the *hrp* genes are present in many pathovars of *P. syringae*. Functional equivalence has been demonstrated for two *hrp* clusters of *P. s. pv. phaseoli-*

*cola* with their corresponding homologues in pathovars *glycinea*, *tabaci*, *angulata* and *savastanoi* (Lindgren et al., 1988; Grimm and Panopoulos, unpublished observations), which share a high degree of total DNA homology with each other. Phenotypically similar mutants have also been obtained in *Erwinia amylovora* (Steinberger and Beer, 1988) and in pathovars of *Xanthomonas campestris* (Staskawicz, personal communication) both of which are phylogenetically distant from *Pseudomonas*. Hybridization analysis with *hrp* probes suggests at least two *hrp* homology groups exist among different phytopathogenic taxa. Homology to the *P. s. pv. phaseolicola* probes appears to be restricted to pathovars of *P. syringae* while the probes from *P. solanacearum* also hybridized to DNA from *Xanthomonas* (Boucher et al., 1987; Lindgren et al., 1988).

Proteins that are present in the wild type strains but are absent from  $Hrp^-$  ( $Hri^-$ ) mutants also have been detected in gels by immunological techniques using whole cell antisera, maxicell analysis of *hrpM* and a coupled transcription/translation system for *hrpS* (Xu et al., 1988; C. Grimm and N. J. Panopoulos, unpublished observations; Mukhopadhyay et al., 1988; Niepold and Huber, 1988; Somlyai et al., 1986). However, there are few clues about the biochemical functions of these proteins and *hrp* genes in general in disease and hypersensitive necrosis development. Baker et al. (1987) reported that the  $K^+/H^+$  exchange reaction observed in cells undergoing HR was associated with *hrp* gene function. The same group also reported that a cosmid clone carrying the *hrp* cluster of a *P. s. pv. syringae* strain imparted HR-like necrosis-inducing ability (but not pathogenicity) in *P. fluorescens* and in *E. coli*. Further genetic analysis in this system may provide important functional information by taking advantage of the wealth of genetic knowledge available in *E. coli*.

Primary sequence data for a *hrp* locus (*hrpS*) of *P. s. pv. phaseolicola* suggested that its protein product is regulatory, as it shared significant homology (41 to 48% identical residues) to a highly conserved domain of several prokaryotic regulatory proteins. These included the nitrogen assimilation regulator protein NtrC, the *nif* gene activator protein NifA, the C-4-dicarboxylate transport regulator protein DctD, and the tyrosine repressor TyrR (Grimm et al., 1989; C. Grimm and N. J. Panopoulos, 1989). The *hrpS* gene was subsequently shown to be necessary for the activation of several other *hrp* genes in *P. s. pv. phaseolicola* that are induced during early stages of pathogenesis and hypersensitive necrosis and also in response to nutritional downshift (C. Grimm and N. J. Panopoulos, unpublished observations; M. N. Mindrinos, L. G. Rahme, and

N. J. Panopoulos, unpublished observations; Rahme et al., 1990). Nucleotide sequencing also predicted that a protein homologous to HrpS is encoded by an adjacent gene, *hrpR*, both in *P. s. pv. phaseolicola* and in *P. s. pv. glycinea* (C. Grimm, N. J. Panopoulos, B. J. Staskawicz, and D. Dahlbeck, unpublished observations). This protein presumably plays a regulatory role but its promoter specificity is not yet known. It further appears that the *hrpS-hrpR* region and an additional locus (*hrpL*) are necessary for the expression of an avirulence gene (*avrB*) of *P. s. pv. glycinea* in response to a nutritional downshift and also in planta (T. V. Huynh, D. Dahlbeck, and B. J. Staskawicz, unpublished observations).

Complete nucleotide sequences for *hrpS* have been determined for three pathovars, *phaseolicola*, *glycinea*, and *savastanoi*. It also has been determined for the *hrpR* gene in the first two and for the unlinked locus, *hrpM*, in pathovars *phaseolicola* and *syringae* (R. Frederick and N. J. Panopoulos, unpublished observations; Mukhopadhyay et al., 1988). The latter locus is structurally unusual in that it contains four large, antiparallel open reading frames in codon-to-codon register over most of their length. Only two of these were shown to be functionally important. One of them encodes an 83 kDa protein with several membrane-spanning segments, and the other encodes a 42 kDa protein which shares homologous stretches with histones. The *hrpM* is the only *hrp* locus known to control bacterial colony phenotype (mucoidy).

The *hrp* cluster of *P. solanacearum* is similar in size to that of other pseudomonads and shows many similar features and some other interesting properties (Boucher et al., 1988a, 1988b). The region is conserved in all 52 strains of the pathogen representing all different races, biovars, and geographical origins. In most strains, possibly all, the cluster is located on a megaplasmid and is deleted entirely in Acr;xr (acridine orange resistant) mutants. These mutants also exhibit methionine auxotrophy, secrete a brown pigment during growth in rich medium, and differ from the wild type in the structure of their lipo- and exopolysaccharides (Drigues et al., 1985). Chemically defined changes in LPS structure have previously been described for a well known class of spontaneous avirulent mutants ("B-one" type) and for mutants selected for resistance to an LPS-specific phage (Hendrick and Sequeira, 1984). The B-one mutants are altered in several other properties (production of fimbriae, attachment to host cells, aerotaxis, motility, and production/secretion of cell wall degrading enzymes). Genes for pilus synthesis and extracellular enzyme production (*egl*) were recently cloned but their relationship to gene carried by

the *hrp* cluster is not yet clarified (Denny et al., 1988b; Roberts et al., 1988; Schell, 1987; Stemmer and Sequeira, 1987). An interesting new development is the identification of a gene encoding a genotype-specific HR-like necrosis eliciting protein from a strain that is pathogenic on potato (Xu et al., 1988). These findings coupled with the functional characterization of the *hrp* cluster should help clarify the seemingly complex genetics and physiology of vascular wilt pathogenesis by *P. solanacearum*.

### Avirulence Genes

The term "avirulence gene" was coined to describe those genes in a pathogen that relate to a product recognized by the host and causing an incompatible reaction, hence no disease. Flor's gene-for-gene hypothesis (Flor, 1971) provided a framework for understanding the genetics of specificity in plant-pathogen interactions. A commonly held interpretation of this hypothesis predicts that specificity between host and pathogen resides in the incompatible interaction which is genetically determined by dominant genes in the host and the pathogen. These genes are generally termed "resistance" and "avirulence" genes, respectively. The cloning of "avirulence" genes from *P. s. glycinea* (Staskawicz et al., 1984) provided an elegant verification of this hypothesis at the molecular level and its applicability to a bacterial pathogen. This study and those which followed (Napoli and Staskawicz, 1987; Tamaki et al., 1988; Keen and Staskawicz, 1988) demonstrated that race-cultivar specific incompatibility, expressed as hypersensitive response in the soybean blight-*P. s. pv. glycinea* system requires the functions of distinct genes (*avr*) for different resistance genes in the host cultivar. Mutational inactivation of a given *avr* gene changes the incompatible interaction (HR) to a compatible one (development of watersoaked lesions) on cultivars that are known or can be presumed to possess a functionally complementary gene for resistance, while the interaction with cultivars lacking such a gene remains the same. Similar changes in reaction type on such cultivars may be obtained when the functionally correspondent *avr* gene in the pathogen is inactivated. Interstrain transfer of a given *avr* gene changes the interaction phenotype in the opposite direction. Thus, both types of analysis produce the result predicted from the gene-for-gene hypothesis.

Avirulence genes have been cloned from several other pathovars of *P. syringae*, including *tomato* (Kobayashi et al., 1989) and *phaseolicola* (Harper et al., 1988; Shintaku et al., 1989), and from several pathovars of *Xanthomonas* (Gabriel et al., 1986; Swanson et al., 1988; Kearney

et al., 1988; Keen and Staskawicz, 1988). Four of them have been sequenced and their products identified (Napoli and Staskawicz, 1987; Ronald and Staskawicz, 1988; Tamaki et al., 1988). However, the functions of Avr proteins are still unclear. Necrosis-inducing activity has been associated with the *avrD* gene, which was cloned from *P. s. pv. tomato* (Kobayashi et al., 1989), when it is expressed from a strong promoter in *E. coli* (N. T. Keen, personal communication). This has not yet been demonstrated for other *avr* genes. Also unclear is the relationship, if any, between the *avrD* necrosis elicitor, the 60 kDa genotype-specific necrosis elicitor of potato described in *P. solanacearum* (Xu et al., 1988) and the K<sup>+</sup>/H<sup>+</sup> exchange promoting substance(s) produced by *P. fluorescens* harboring the *P. s. syringae* *hrp* cluster (Baker et al., 1987). Detailed characterization of the active molecular species in these cases should be forthcoming.

As discussed earlier in this chapter, the phenotypic expression of HR by *Pseudomonas* requires *hrp* gene function in addition to *avr*. The existence of Hrp<sup>-</sup> mutants, particularly in pathovars from which *avr* genes were cloned, reveals a genetic dependence of *avr*-mediated incompatibility on *hrp* gene function. This dependence is specifically shown by the fact that the hypersensitive necrosis elicited by *P. s. pv. glycinea* race O on cultivars Acme and Harosoy, where the only functionally relevant *avr* genes are *avrC* and *avrB*, respectively, is no longer elicited when the *hrp* region is not functional (Lindgren et al., 1988). This suggests that *avr* and *hrp* genes or their products interact with each other, either directly or indirectly, and that these interactions are an important part of the chain of events leading to the elicitation of HR and incompatibility. The nature of these interactions is largely unexplained except for one *avr* gene (*avrB*) whose transcription requires the function of certain *hrp* genes in *P. s. pv. glycinea* (T. V. Huynh, D. Dahlbeck, B. J. Staskawicz, unpublished observations). Whether similar regulatory requirements exist for other avirulence genes is not known. However, other types of *avr-hrp* gene interactions must also occur. For example, expression of *avrB* does not require the functions of the majority of *hrp* genes, which are nevertheless necessary for necrosis development (Huynh et al., 1989).

Avirulence genes were recently shown to impart new pathogenic specificities of *Pseudomonas* on non-hosts (species and genus level). (Kobayashi et al., 1989; Whalen et al., 1988) For example, specific cosmid clones from *P. s. pv. tomato* changed the compatible interaction of *P. s. pv. glycinea* to incompatible on certain soybean cultivars but not on others (Kobayashi et al., 1989). *P. s. pv. tomato* was later

shown to contain an homologue of the *P. s. pv. glycinea avrA* gene which could impart avirulence on *P. s. pv. tabaci* but not on pathovars *pisi*, *phaseolicola* and *lachrymans*. Similar results were obtained through transfer of genomic libraries or specific avirulence genes between *Pseudomonas* and *Xanthomonas* (Whalen et al., 1988). These findings have a very interesting implication, namely, that *avr* genes (i.e. genetically dominant negative factors) are a common class of host range determinants in non-host/pathogen interactions.

### Toxins

Several pathovars of *P. syringae* produce phyto-toxins (Durbin, 1981; Mitchell and Bielecki, 1977; Mitchell, 1984). Chemical structures have been established for four of these toxins as well as rhizobitoxin which was recently reported as being produced by *Pseudomonas andropogonis* (Daly and Deverall, 1983; Durbin, 1981; Mitchell, 1984, 1988; Moore et al., 1984). Known sites of action include amino acid biosynthetic pathway enzymes (e.g. glutamine synthetase and ornithine carbamoyltransferase for the active moieties of tabtoxin and phaseolotoxin, respectively), chloroplast RNA polymerase for tagetoxin, cystathionase for rhizobitoxin, and certain protein kinases of plant and other eukaryotic cells for syringomycin (Abdel-Ghany et al., 1988; Bidwai and Takemoto, 1987; Gilchrist, 1983; Langston-Unkefer et al., 1987; Mitchell, 1984; Tam and Patil, 1972; Templeton et al., 1985). Several of these toxins are capable, in purified form, of reproducing one or more aspects of disease symptomatology attributed to them, usually chlorosis or necrosis. The causal relationships between the effect of the toxin on the target site and expression of disease symptoms are less clear (Gilchrist, 1983; Mathews and Durbin, 1987; Mitchell, 1984; Turner and Mitchell, 1985; Turner and Taha, 1984).

In contrast to the phytotoxins produced by fungal pathogens, which are host-specific, the *Pseudomonas* toxins are active against plants on which their producers do not cause disease. Some of these toxins are also active against microorganisms. The latter property has been exploited by researchers to develop convenient bioassays that have expedited purification and mode of action and genetic studies on these toxins (Gasson, 1980; Gross et al., 1977; Mitchell et al., 1980; Morgan and Chatterjee, 1985; Peet and Panopoulos, 1986; Staskawicz and Panopoulos 1979; Turner and Taha, 1984; Xu and Gross, 1988a). Genetic studies have been reported for five *Pseudomonas* toxins. Genes involved in toxin production have only been detected by hybridization in organisms already known to

produce these substances, suggesting possible uses as diagnostic probes. For example, a *tox* gene fragment from *P. phaseolicola* has proven useful as a diagnostic probe (Schaad et al., 1989) and similar possibilities exist for other pathogens.

Whether toxins constitute "pathogenicity" or "virulence" factors (sensu Yoder 1980) has been actively debated among plant pathologists. As with other pathogenicity or virulence genes, the operational criteria for making such distinctions are not uniformly applied by investigators, and disease symptomatology, usually studied only in the laboratory, differ enough from one case to another to confuse the picture. Nevertheless, some *Pseudomonas* toxins clearly belong to the second group, one example being phaseolotoxin. Mutants of *P. s. pv. phaseolicola* that do not produce phaseolotoxin show identical growth kinetics in the plant as do wild-type strains (Patil et al., 1974; Peet et al., 1986). Other *Pseudomonas* toxins that have been examined in this respect (e.g. coronatine, syringotoxin) appear to play a role in pathogen multiplication in the plant since *Tox-* mutants of *P. s. pv. tomato* and *P. s. pv. syringae* (citrus strain) were attenuated in virulence (Bender et al., 1987; Xu and Gross, 1988a).

Two *Pseudomonas* toxins, syringomycin and syringotoxin, are cyclic peptides containing one or more nonprotein amino acids and are presumed to be synthesized by enzyme systems analogous to those involved in the synthesis of cyclic peptide antibiotics. Large-molecular-weight proteins or protein complexes have been identified that are thought to be toxin synthetases (Morgan and Chatterjee, 1988; Xu and Gross, 1988b). Two other toxins, tabtoxin and phaseolotoxin, are synthesized and secreted from the cells as linear peptides but are hydrolyzed in the plant to a nonpeptide form (tabtoxinine- $\beta$ -lactam and N-[phosphosulphinyl] $\delta$  alanine, abbreviated Psorn, respectively) which is considerably more active against the target enzyme. Cleavage by plant peptidases potentiates the action of these toxins *in vivo*. This strategy may be a means of exploiting general oligopeptide permeases for the "illicit" transport at least into microbial cells (Staskawicz and Panopoulos, 1979), and may also be part of the strategy for self-protection in the producer strains (Durbin and Langston-Unkefer, 1988). However, both *P. s. pv. tabaci* and *P. s. pv. phaseolicola* have additional self-protection mechanisms: the first through biochemical modification of its glutamine synthetase to a form not sensitive to the toxin and perhaps also through formation of a tabtoxin-hydrolyzing periplasmic aminopeptidase, and the second through a duplicate ornithine carbamoyltransferase that is much less

sensitive to phaseolotoxin and Psorn and is produced only during periods of toxin synthesis (Durbin and Langston-Unkefer, 1988; Ferguson et al., 1980; Knight et al., 1986, 1987; Levi and Durbin, 1986; Peet and Panopoulos, 1988; Staskawicz et al., 1980).

## Phytohormones

Phytohormones represent the only group of low-molecular-weight bioactive substances besides the toxins for which a role in pathogenesis by *Pseudomonas* is rigorously established. A useful conceptual distinction between toxins and phytohormones is that the latter are also produced by the host. A role for phytohormones is usually suspected when pathogens induce growth abnormalities in the host, examples being the crown gall, hairy root, and olive knot diseases. However, surveys (Akiyoshi et al., 1987; Fett et al., 1987) based on cytokinin radioimmunoassay and a combination of analytical techniques for detection of indoles demonstrated for IAA and related indoles showed that other pathogens that do not cause hypertrophic symptoms on their hosts also produce these substances. Thus, nine different plant pathogenic pseudomonads produce IAA and additional indole compounds in media that have been supplemented with tryptophan. *P. solanacearum* and *P. s. pv. syringae* also synthesize exceptionally high amounts of IAA both in the presence and absence of exogenous tryptophan (Fett et al., 1987; Phelps and Sequeira, 1967). A role of hormones in either vascular wilt or in the brown spot disease has not been established. IAA has numerous other effects on plant cells which may favor bacterial multiplication in the absence of hypertrophy.

There is definitive evidence for the involvement of IAA in the interaction between *P. s. pv. savastanoi* and two of its hosts (Morris, 1986; Nester and Kosuge, 1981; Schroder, 1987). The pathogen synthesizes the hormone from tryptophan in two steps catalyzed by tryptophan monooxygenase and indoleacetamide hydrolase, the products of the *iaaM* and *iaaH* genes, respectively. The functions of *iaaM* and *iaaH* are essential for the hyperplastic response. Strains of *P. s. pv. savastanoi* that incite hyperplasias on oleander and olive differ genetically from strains that are only pathogenic on olive in two respects: first, they carry the *iaaM* and *iaaH* genes on plasmids (pIAA) rather than on the chromosome; second, they possess an additional gene, *iaaL*, that encodes an IAA-lysine conjugating enzyme, which apparently regulates the IAA-pool size in oleander strains. The *iaaL* gene also is carried on pIAA plasmids and behaves as a positive host range determinant of gall-forming ability on ole-

ander. When it is inactivated, only minor swellings are formed on this host (Glass and Kosuge, 1988) rather than typical galls. Unlike the crown gall disease, there is no evidence for *iaa* gene transfer to plant cells by *P. s. pv. savastanoi*.

Several groups (Akiyoshi et al., 1987; Morris, 1986; Surico et al., 1985) have reported that *P. s. pv. savastanoi* and *P. solanacearum* produce cytokinins. There is diversity in both the type of cytokinins and the amounts produced by different strains. Except for *trans*-zeatin riboside, which acts as a virulence factor in *P. s. pv. savastanoi* (Roberto et al., 1988), the role of these substances in symptom expression and the basis of the diversity of the cytokinin spectra have not yet been established. In *P. s. pv. savastanoi* 1006 (an olive strain), a plasmid-borne gene (*ptz*) encodes isopentenyl transferase activity when cloned in *E. coli*. A gene designated *tzs* was also cloned from *P. solanacearum* K60, but its location and linkage were not determined (Akiyoshi et al., 1987).

The deduced amino acid sequences of *P. s. pv. savastanoi iaaM*, *iaaH* and *ptz* gene products share significant similarity to the Ti plasmid genes for phytohormone biosynthesis (*tms-1* and *tms-2* for IAA and *tzs* and *ipt* gene for cytokinin). Interesting speculations have been put forward both about the possible origins and about the evolutionary relationships of these genes. For example, Comai and Kosuge (1983) suggested that the *iaa* genes of *P. savastanoi* originated from genes for utilization of exogenous tryptophan under saprophytic conditions where IAA served as an intermediate. A simple loss of auxin degradation would lead to accumulation of the hormone. Schell (1986) and Yamada et al. (1985, 1986) further speculated that the genes for hormone biosynthesis found on Ti plasmids may have originated from those of *P. s. pv. savastanoi*. The acquisition of eukaryotic expression signals may be viewed as a highly refined improvement. This would enable a vastly expanded host range and transfer of the biosynthetic cost of hormone production from the bacterial cell to the plant (Morris and Powell, 1987; Weller and Schroder, 1987). The gene acquisition proposal further suggests that the phytohormone biosynthetic capacity of *P. s. pv. savastanoi* evolved before *A. tumefaciens* acquired the ability to form hormone-autotrophic tumors. The degree of sequence similarity between the phytohormone genes and their products (about 50%) would predict that the proposed gene transfer event occurred very early in prokaryote evolution (Ochman and Wilson, 1987). Recently, Sekine et al. (1989) suggested a common origin for *iaaH*, *tms-2*, and a gene (*bam*) encoding IAA hydrolase that was cloned from *Bradyrhizobium japonicum*. Since we know little about the origin

and evolution of plant pathogens, these hypotheses are intriguing and invite more extensive and detailed phylogenetic and molecular evolutionary consideration.

### Ice Nucleation Genes and Proteins

Ice nucleating *Pseudomonas* (*Ina*<sup>+</sup>) are among the most efficient ice nucleators at temperatures above -10°C (Lindow, 1978; Lindow, 1983). Three species of this taxon contain ice nucleating members: *P. syringae* (various pathovars), *P. fluorescens* (biotype G), and *P. viridiflava* (several strains). The study of bacterial ice nucleation received particular attention in recent years because: 1) the role of ice nucleating bacteria, particularly *P. syringae* as agents of frost injury to plants had been well established in the 1970's; 2) a broad range of potential applications of ice nucleators in various industries exists; 3) the phylloplane, which these bacteria normally occupy, is a relatively simple environment and, therefore, more easily amenable to modification than other environments (e.g., the rhizosphere); 4) the phenomenon itself has inherent scientific interest; and 5) an important development, namely the cloning of ice-nucleation genes, occurred in 1982 (Orser et al., 1983). This made possible the analysis of the molecular, genetic and biochemical basis of bacterial ice nucleation by new high-resolution methods.

It is now firmly established that a family of large and unusual proteins (ice nucleation proteins, 118 kDa or larger) are a key component of bacterial ice nuclei (Corotto et al., 1986; Green and Warren, 1985; Lindow et al., 1989; Warren, 1987; Wolber et al., 1986; Warren, 1987). Predicted amino acid sequences of two such proteins (*InaZ* and *InaW*, from *P. syringae* and *P. fluorescens*, respectively) revealed a very interesting structure from the standpoint of ice nucleation function. Both proteins consist for the most part of about 120 tandem repeats of a "consensus" octapeptide with alternative periodicities (e.g., 16 or 48 amino acids) superimposed on this basic pattern (AGYGSTXTAGXXSSLI AGYGSTQTAGXXSXLT AGY-GSTQTAQXXSXST, the letters specifying amino acids according to the standard single-letter code, X being a variable residue). Although not unique among proteins, the repeat structure suggests itself as being central to the ice nucleation function. Several repeat domains may collectively form a catalytic surface of some critical size which effectively orders water molecules in an ice-like lattice. Although crystallographic data are not yet available, tertiary structures of ice nucleation proteins have been proposed (Warren et al., 1986; Warren, 1987).

The sequence similarity between the *inaZ* and *inaW* genes leaves little doubt that they have a common origin. It has been suggested that these genes may have evolved by amplification of an eight-codon motif (Green and Warren, 1985; Warren, 1987). It is interesting to know whether this applies to other similar genes. Nucleotide sequence data for the ice nucleation genes from *Erwinia herbicola* and *Xanthomonas translucens*, which are phylogenetically distant from the pseudomonads, may be informative as to whether the scattered distribution of ice nucleation genes among the *Pseudomonas* and other eubacterial evolutionary lines reflects common ancestry or is a result of lateral transfer.

The nature, composition, and other properties of bacterial ice nucleation sites have been analyzed at considerable depth by a variety of methods. *Pseudomonas* ice nuclei are cell-bound and contain both protein and lipid since treatment of partially purified outer membranes of *P. syringae* with either proteinase K or various delipidating agents leads to significant loss of ice nucleation activity, which can be subsequently reconstituted by addition of phospholipid (Govindarajan and Lindow, 1988a). There is one unconfirmed report (Kozloff et al., 1984) that phosphatidyl inositol might be a component of ice nuclei. The physical size of ice nucleation sites has been determined in situ, both in *P. syringae* and in *E. coli* expressing ice nucleation proteins, by gamma-ray inactivation analysis (Govindarajan and Lindow, 1988b). Nucleation sites are very large and show a logarithmic relationship between size and temperature at which they are active. For example, sizes of about 9 and 620 kDa correspond to nuclei that are active at or below  $-2^{\circ}\text{C}$  and  $-9^{\circ}\text{C}$ , respectively. The minimum mass of a functional ice nucleus, active only between  $-12$  and  $-13^{\circ}\text{C}$ , was about 150 kDa, which is in the size range of the ice protein monomer. Ice nuclei are predominantly associated with the outer bacterial membrane (Lindow et al., 1989), although aggregates that are not membrane-associated but contain ice nucleation protein are found in cells that overproduce the protein (Deininger et al., 1988; Wolber et al., 1986).

Ice nucleation protein monomers presumably associate with other such molecules and with lipids but little is known about the physicochemical nature of these associations. For example, chemical cross-linking experiments indicate that ice protein monomers have other such molecules as nearest neighbors (Govindarajan and Lindow, unpublished observations). The relationship between ice protein concentration (P) and ice nucleus activity per cell (N) in *E. coli* and *P. s. pv. phaseolicola* is nonlinear (Lindgren et al., 1989; Southworth et al., 1988). When activity is

measured at  $-9^{\circ}\text{C}$  the activity of N increases according to the second power of P over a large range of values up to about 0.4 nuclei/cell. High-power relationships apply when N is measured at warmer temperatures.

Practical and research applications connected with ice nucleating *Pseudomonas* and the ice nucleation genes are discussed in detail in several reviews (Lindow, 1987; Lindow and Panopoulos, 1988; Warren, 1987), but a few representative examples may be mentioned briefly here. One is the use of nonviable Ina<sup>+</sup> cells in snow-making technology for ski resorts. Another is the use of a promoterless ice nucleation gene as a sensitive "reporter" of bacterial gene expression (several hundred thousand-fold more sensitive than the conventional  $\beta$ -galactosidase reporter) (Lindgren et al., 1989). A third is the use of ice nucleation genes and/or proteins in diagnostic technology (Warren, 1987). Finally, site-directed deletion of the ice nucleation gene from epiphytically competent nonpathogenic strains of *P. syringae* and *P. fluorescens* ("iceminus" and Frostban;rm, respectively) was used to generate prototype biological control agents of frost injury to plants, the first "genetically engineered" bacteria to be released outside the laboratory under Environmental Protection Agency (EPA) permit in the USA (Lindow and Panopoulos, 1988). Such strains are presumably free of outside mutations and thus permit more definitive analysis of bacterial interactions in the phylloplane (Lindow, 1987).

## Habitats of Phytopathogenic and Related Plant-Associated Pseudomonads

Plant pathogenic and plant-associated pseudomonads are found in a multiplicity of environments. Crosse (1968) defined four categories of bacterial pathogens in relation to their principal habitat and mode of survival: 1) pathogens with no soil phase; 2) pathogens with a transitory or ephemeral soil phase; 3) pathogens with a protracted soil phase; and 4) those with a permanent soil phase. The different taxonomic groups of the bacterial pathogens fit well into these categories as there is a strong relationship between taxonomic groupings and the kinds of diseases they cause. There also is a good correlation between groupings and disease epidemiology (Schroth et al., 1978). The great majority of phytopathogenic pseudomonads are foliar pathogens. They do not survive long in soil if not associated with the host or host residues.

Pseudomonads constitute a substantial part of the microflora in the rhizosphere and rhizoplane



and some may on occasion cause some damage (Schippers et al., 1987; Suslow and Schroth, 1982). These pseudomonads are primarily saprophytes but are excellent competitors on plant parts. They also are commonly found in the endo-rhizospheres (Foster et al., 1983); such strains could be placed into Crosse's group 4.

### Foliar Pathogens

The great majority of the foliar pathogens, approximately 50 or more when including pathovars and species, belong in habitats 1 and 2 (no soil phase or a transitory soil phase). Most of them are pathovars of *P. syringae*. As reviewed by Schroth et al. (1978) and Schuster and Coyne (1974), most foliar pathogens that have been studied have the capability of surviving in plant residues up to a year when the host plant is plowed under. However, the duration of time that the pathogen survives greatly depends on soil temperature and moisture. Few if any will survive long when the environment is warm and moist and ideal for decomposition of plant debris. Some pathogens appear to colonize plant roots for short periods of time (Schneider and Grogan, 1977). However, there is little evidence that any of these strictly foliar pathogens survive in soil when not associated with host tissues.

*P. syringae* and its many pathovars are closely associated with the living plant and the mechanisms of survival are ideal for perpetuation. For example, stone and pome fruit pathogens such as *P. syringae* pv. *syringae*, *P. s.* pv. *savastanoi* and *P. s.* pv. *morsprunorum* survive in lesions, cankers, tumors, or sometimes as leaf epiphytes. Therefore, there usually is available inoculum present for dissemination to infection courts when environmental conditions suitable for infection occur. There is a great amount of information available on the epiphytic activities of these and other foliar pathogens: *P. s.* pv. *syringae* (Ercolani et al., 1974; Lindow et al., 1988), *P. s.* pv. *savastanoi* (Ercolani et al., 1971; Wilson, 1935), *P. s.* pv. *lachrymans* (de Lange and Leben, 1971), *P. s.* pv. *glycinea* (Leben et al., 1968), *P. s.* pv. *persicae* (Gardan et al., 1972) and others (Leben, 1974). These bacteria in general can be characterized as follows: they have an epiphytic growth phase (Hirano and Upper, 1983), many are seed-borne, others maintain a permanent plant-associated relationship (epiphytic growth or incipient infections), and all can be disseminated by water, insects, and other animals. They cause blights, cankers, overgrowths, leaf and fruit spots, wilts, and rots (*P. viridiflava*). They are motile, which enhances survival by enabling them to explore large areas of the microenvironment (Leben et al., 1970) and to respond quickly

to favorable and unfavorable chemotactic stimuli (Barachini and Sherris, 1959; Cuppels, 1988; reference is not an exact match Kock, 1971; Smith and Doetsch, 1968). The importance of flagellar motility was shown by Panopoulos and Schroth (1974) using isogenic pairs of motile and nonmotile strains; twelve times as many lesions were caused by motile strains as compared to their nonmotile counterparts. There is no evidence that motility affects systemic activities.

Although foliar bacterial pathogens are disseminated by rain, much of the inoculum remains on leaves since it is not easily washed off plant surfaces. This presumably is related to the electrical charge of the cell surface. Factors affecting motility of pseudomonads have not received much attention. It generally is known that substances such as amino acids and carbohydrates serve as attractants (Chet et al., 1973).

### Soil-Borne Pathogens

Only a relatively few bacterial pathogens can be placed into category 3 (diseases with a protracted soil phase). However, it is doubtful if most would survive long in fallow soil and there is little evidence that any of them compete well when colonizing residues or nonliving plant materials. Examples of soil-borne bacteria are the fluorescent pathogens *P. tolaasii*, *P. marginalis* (synonym *P. fluorescens* biovar 2) and *P. aeruginosa*, and the nonfluorescent pathogens *P. cepacia*, *P. gladioli* pv. *gladioli*, *P. gladioli* pv. *alliiicola*, *P. plantarii*, and *P. solanacearum*. *P. aeruginosa* is commonly isolated from soil (Green et al., 1974), and *P. marginalis* seems to be ubiquitous in soil and often as an internal resident of plant tissues. The latter bacterium has not been well defined taxonomically, nor has it been examined carefully as a plant pathogen. Disease appears to be triggered by conditions of high moisture such as when sprinkling lettuce. There also have not been comprehensive studies on *P. tolaasii* (mushroom disease) (Wong and Preece, 1979), *P. g.* pv. *gladioli*, and *P. g.* pv. *alliiicola*. There are a number of biovars and races of *P. solanacearum* as previously discussed, and they vary greatly with respect to the types of diseases they cause and modes of survival. Race 1, which infects solanaceous and other plants, appears to survive well in soil probably because of its ability to colonize roots of many plants. Race 2 (the banana and heliconia strains) does not survive long in soil (Buddenhagen, 1965).

### Pathogens of Humans

*P. aeruginosa* and *P. cepacia* are interesting in that they are among the few plant pathogens that

have been implicated as causing diseases of both plants and animals. *P. aeruginosa* is an important nosocomial pathogen of humans (reference is not an exact match von Graevenitz, 1977) and is particularly significant in burn wards or when patients are compromised by other diseases such as cancer. It is a quasipathogen of plants (Cho et al., 1975; Green et al., 1974; Schroth et al., 1977), causing damage only when plants are subjected to stress such as high temperatures and moisture. Many strains isolated from plants and soil appear to be identical to a number of the major pyocin types of clinical origin (Kominos et al., 1972; Schroth et al., 1977). Lebeda et al. (1984), working with both plants and animals, concluded that both clinical and plant strains of *P. aeruginosa* were pathogenic for animals but not soil strains. There are only two cases where *P. aeruginosa* has caused a disease in the field and/or in storage; Cother et al. (1976) and Gupta et al. (1986) reported that the bacterium caused a severe case of onion bulb rot. However, others (Knösel and Nimitan, 1976) have questioned that *P. aeruginosa* is a cause of plant disease.

The dual role of *P. cepacia* as a plant and animal pathogen is not clear primarily because of the unsettled taxonomy reference is not an exact match Gonzales and Vidaver (1979) reported, from a study of a relatively few strains isolated from animals and plants, that they differed in a number of characteristics. It is important to know if these differences would be maintained if the study were done with a larger assemblage of strains. *P. cepacia* is a serious and common pathogen of onion (Kawamoto and Lorbeer, 1976). It also has been reported to be a common rhizosphere microorganism and has been used as a biocontrol agent against various fungal plant pathogens (Weller, 1988). With respect to humans, it causes serious diseases of compromised patients (Bassett et al., 1970; Bottone et al., 1975; Carson and Petersen, 1975; Ederer and Matsen, 1972; von Graevenitz, 1977), especially when it is a contaminate of fluids or substances that are injected into the bloodstream. The unsettled taxonomy of *P. cepacia* is another case that suffers from the general inactivity in the field of systematics.

### Host Specialization

Many of the phytopathogenic pseudomonads have been characterized as having narrow host ranges. For example, most of the *P. syringae* pathovars were thought to attack only closely related species of plants. However, exceptions are known. Pathovar *tabaci* is reported (Kennedy and Tachibana, 1973) to cause a disease of both tobacco and soybean, some strains

of pv. *phaseolicola* infect both mulberry and soybean (Schroth et al., 1971), pv. *tomato* has been isolated from diseased stone fruits (Arsenijerik and Hildebrand, personal observations) and, as previously stated, *P. maculicola* and *P. antirrhini* can infect tomato (Hildebrand et al., 1984). *P. s. pv. syringae* has a large host range and is a common epiphyte on many plants.

There is no doubt some host specialization with pv. *syringae*, but this has not been well worked-out because of the enormity of the task, including the complex matter of defining pathogenicity, as discussed subsequently. Pathovar *syringae* strains associated with blights and blights of stone and pome fruits, citrus (Gross et al., 1977), legumes (Saad and Hagedorn, 1972), and various other plant hosts (Ott, 1974; Sands et al., 1977; Shane and Baumer, 1987; Smith and Mansfield, 1981; Surico and DeVay, 1981) appear to have some different pathological properties; for example, the legume strains are clearly host-specific and the citrus strains can be distinguished by certain phenotypic tests (Hildebrand, unpublished observations), the production of syringomycin and syringotoxin (Gonzalez et al., 1981), and both can be distinguished by southern hybridization and RFLP analysis (Hendson and Hildebrand, unpublished observations).

Host specialization has only been intensively studied with a few pathovars. *P. s. pv. phaseolicola*, the cause of halo blight of bean, was ideal for pathogenicity studies because of the ease of inoculating a large number of herbaceous plants and obtaining fast, relatively unequivocal results (Schroth et al., 1971). Pathovar *phaseolicola* attacks *Phaseolus vulgaris* L. (common bean), *P. limensis* Macf. (large-seeded lima bean), and *P. lunatus* f. *macrocarpus* (Benth.) Van Ess. (small-seeded lima bean), whereas the closely related pathogen *P. glycinea* attacks *Glycine max* (L.) Merr. (soybean). The diversity and plasticity of pathovars is exemplified by pathovars *phaseolicola* and *glycinea* in that a few strains of each pathovar attack the primary host of the other and both pathovars and *P. s. pv. mori* produce similar symptoms on lima bean.

*P. viridiflava*, *P. cichorii*, and *P. marginalis* appear to have broad host ranges (Fahy and Lloyd, 1983; Pieczarka and Lorbeer, 1975; Sampson and Hayward, 1971). These pathogens cause similar symptoms on a wide range of vegetables and appear to be favored by conditions of high moisture. *P. viridiflava* causes a watery rot of foliar parts of many plant such as tomato, peach, pumpkin, crucifers, and cowpea. *P. cichorii* is a common lettuce pathogen but has been found on chicory, endive, clover, cauliflower, poppy, celery, tobacco and other plants. The ill-defined *P. marginalis* has been attributed as the cause of various field and storage rots of

vegetables. None of these pathogens have been well-researched.

*Pseudomonas solanacearum* is a particularly interesting pathogen with respect to its ecological and pathological characteristics. Race 1 infects solanaceous and other plants, race 2 infects banana and heliconias, and race 3 infects potato. Each race consists of numerous known and unknown pathovars.

### Pathogenicity Tests

Experienced phytobacteriologists generally have little problem in evaluating the potential of a pathogen to cause disease or in assessing virulence. However, this general area represents one of the greatest “pitfalls” in phytobacteriology. An understanding of the problems and errors in interpretation that often are associated with assessing host ranges and pathogenicities is critical. This affects decisions made by regulatory agencies, which in turn affect such activities as quarantines and microbial release policies. It also may have a major impact in formulating conclusions when making a molecular analysis of characteristics associated with virulence. The ability of many bacterial plant pathogens, particularly those causing foliar and wilt diseases, to cause an assortment of reactions on nonhost plants has contributed to many misunderstandings and erroneous claims with respect to host ranges and virulence. It frequently is overlooked when conducting plant pathogenicity tests that massive dosages of bacteria ( $>10^7$ ) when injected into a plant with a syringe or other similar means will cause a necrotic hypersensitive response (Klement, 1963) and a variety of other reactions, depending upon the plant (Hildebrand et al., 1988). Massive inoculations of bacteria into nonhosts result in bacterial multiplication for a limited period of time (Ercolani and Crosse, 1966; reference is not an exact match Király et al., 1977) with the production of metabolites. This causes injury to the host manifested by a variety of symptoms. This type of host-solicited reaction should not be confused with those resulting from a true host-pathogen relationship. Such artificially contrived host responses do not occur normally in the field nor do the bacteria spread to other contiguous tissues as with a true disease. When in doubt about the validity of results from a pathogenicity test, it is recommended that the experiment be repeated using an inoculation technique that most closely simulates bacterial inoculation in nature. Thus, when working with a leafspotting bacterium, the leaves of plants should be sprayed gently with an inoculum of  $10^5$  to  $10^7$  and the plants placed in a moist chamber for 12–24 hrs to enable the bacteria to invade the stomates as they would in nature. Although this

is a greater dosage of bacteria than would be expected to occupy an infection court in nature, infections frequently do not occur with laboratory-grown inoculum at lesser densities.

Another commonly made error in conducting pathogenicity tests is to grow plants in unfavorable environments or under conditions that natural host resistance mechanisms are attenuated. For example, plants grown in greenhouses or growth chambers with inadequate lighting, or at abnormal temperatures, often lose their natural resistance to pathogens. Experimental designs that greatly favor multiplication of the pathogen, such as using detached plant parts or “compromising” the host, should be considered suspect. Under such conditions, pathogens often attack plants which normally would not be infected. In short, pathogenicity tests require the exercise of good judgement concerning the relevancy of the experimental design. Accordingly, some investigators have called for standardized inoculation tests. However, this is neither reasonable nor practical when considering the range of diseases and modes of pathogenesis.

### Isolation of Phytopathogenic *Pseudomonads*

Experienced diagnosticians generally use relatively few media for isolating most bacterial plant pathogens. Most media used are not selective since highly selective media generally are somewhat toxic and the percentage recovery of cells is much less compared to standard rich media. In addition, selective media tend to discriminate for certain populations within a taxon since media frequently are designed without testing a broad representation of strains. Consequently, investigators often note that selective media are more useful in some geographical areas than others.

The choice of using a general medium such as King’s medium B over a selective medium depends greatly upon the nature and condition of the isolation material. For example, it usually is not necessary to use selective media when isolating from lesions or diseased plant material if the infections are relatively fresh and secondary invaders have not colonized the tissues. With good material, the pathogen is usually the dominant bacterium on the isolation medium. Experienced workers become expert at recognizing bacterial plant pathogens by colony characteristics. When there is a question about identification, there are a number of relatively quick determinative tests that may be used as a check for authenticity. For example, the 30-second oxidase test (Hildebrand et al., 1988; Sands et al.,

1970) quickly differentiates between most fluorescent pseudomonads that cause foliar diseases and other fluorescent pseudomonads including all saprophytes which are oxidase positive. The one exception is the oxidase-positive, arginine dihydrolase-negative *P. cichorii*, which causes leaf spots of several plant species.

Selective media are helpful or necessary when isolating from plant residues, soil, or material when there are many other microorganisms present. Bacterial plant pathogens have relatively slow doubling times compared to many soil saprophytes and thus they are easily inhibited on rich media by faster growing bacteria (Hildebrand and Schroth, 1972).

For more detailed information on the various media that have been described for phytopathogenic bacteria including the determinative tests used to identify them, the reader is advised to consult Fahy and Persley (1983), Lelliott and Stead (1987), and Schaad (1988). We list here some media which are commonly used for isolating pseudomonads.

### Isolation under Nonselective Conditions

King's medium B (King et al., 1954) is the preferred medium for isolation of bacterial plant pathogens because of its nondiscriminatory characteristics and since most fluorescent pseudomonads fluoresce on the medium. Even when the pathogen is suspected to belong to another genus, medium B still is used at least as one of the media, since most pathogens grow well on it and there is always the chance that the pathogen may indeed be a pseudomonad. Most commonly, several media may be used when isolating bacterial pathogens. Some pseudomonads such as *P. cepacia* and *P. gladioli* that do not fluoresce produce other distinctive pigments that help their identification. As previously noted, there are a few pseudomonads that fluoresce better on the media of Garibaldi (1967) and Luisetti et al. (1972) than on King's medium B.

#### King's Medium B for Nonselective Isolation and Pigment Production of *Pseudomonas* (King et al., 1954)

Proteose peptone No. 3 (Difco)	20.0 g
Glycerol	15.0 ml
K <sub>2</sub> HPO <sub>4</sub> (anhydrous)	1.5 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	1.5 g
Agar (Difco)	20.0 g
Distilled water	1.0 liter

Adjust pH to 7.2

### Semi-Selective Media

M-71 Medium (Leben, 1972) for *P. syringae* pv. *glycinea* and Various Other Pathovars of this Group

Glucose	5.0 g
Peptone	10.0
Casein hydrolysate	1.0 g
Cycloheximide	0.05 g
Triphenyl tetrazolium HCL	0.05
Boric acid	1.0 g
Agar	20.0 g
Distilled water	1.0 liter

Although this medium was designed primarily for the soybean bacterial pathogen, it appears to work well for many other pathovars of the *P. syringae* group.

#### King's Medium B plus Cephalexin and Cycloheximide (KCB) (Mohan and Schaad, 1987) for Isolation of *P. syringae* pv. *syringae* and *P. s.* pv. *phaseolicola*

For KBC, the following ingredients are added to 900 ml of King's medium B (made with ingredients for a liter):

Boric acid, autoclaved 1.5% aqueous solution	100.0 ml
Cephalexin (stock solution of 10 mg/ml distilled water)	8.0 ml
Cycloheximide (stock solution of 100 mg/ml 75% methanol)	8.0 ml
Agar	16.0 g

#### Modification of Sucrose Peptone Medium (MSP) (Mohan and Schaad, 1987) for Isolation of *P. syringae* pv. *phaseolicola*

Sucrose	20.00 g
Peptone	5.00 g
K <sub>2</sub> HPO <sub>4</sub>	0.50 g
MgSO <sub>4</sub>	0.25 g
Bromthymol blue (1.5% w/v in 20% ethanol)	1.00 ml
Agar	20.0 g
Distilled water	1.0 liter

Adjust pH to 7.2–7.4, autoclave and cool to 45°C, and then add:

Vancomycin (10 mg/ml aqueous solution)	1.0 ml
Cephalexin (stock solution of 10 mg/ml in water)	8.0 ml
Cycloheximide (stock solution of 25 mg/ml in 12.5% methanol)	8.0 ml

#### Acetamide Medium (Hedberg, 1969) for Isolation of *P. aeruginosa*

The following ingredients are added per 1 liter of water:

NaCl	5.0 g
MgSO <sub>4</sub>	0.2 g
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	1.0 g
K <sub>2</sub> HPO <sub>4</sub>	1.0 g
Acetamide	20.0 g
Agar	20.0 g

Final pH after autoclaving is 6.7

Acetamide medium is excellent for isolating *P. aeruginosa* from plant material and soil (Cho et al., 1975; Green et al., 1974), especially when incubating the cultures at 38°C. Enrichment broth for detecting small populations in soil is made by omitting the agar and incubating shake cultures at 38°C. After 3 days of incubation, an

aliquot of the suspension was plated on acetamide agar.

Trypan Blue Tetracycline (TBT) Medium (Hagedorn et al., 1987) for *P. cepacia*

Glucose	20.0 g
L-Asparagine	1.0 g
NaHCO <sub>3</sub>	1.0 g
KH <sub>2</sub> PO <sub>4</sub>	500.0 mg
MgSO <sub>4</sub> · 7H <sub>2</sub> O	100.0 mg
Trypan Blue	50.0 mg
Tetracycline	20.0 mg
Agar	20.0 g
Distilled water	1.0 liter

pH was adjusted to 5.5 with 10% phosphoric acid (4 ml/liter). The tetracycline was filter-sterilized and added after autoclaving. For low soil dilutions it was recommended that crystal violet, 5 mg/l, and filter sterilized nystatin, 50 mg/l, be added.

Tetrazolium Chloride (TZC) Medium for Isolating *Pseudomonas solanacearum* (Kelman, 1954)

Peptone	10.0 g
Casein hydrolysate (Difco)	1.0 g
Glucose	5.0 g
Oxoid Agar No. 3	12.0 g
Distilled Water	1.0 liter

Adjust pH to approximately 7.0, autoclave, and add to the molten agar (approximately 55°C) filter-sterilized 1% 2, 3, 5-triphenyl tetrazolium chloride to give a final concentration of 0.005%. The medium is usually stored in small bottles for future use.

TPC medium is very good for differentiating virulent from avirulent mutants which readily occur in culture. The virulent wild type forms irregular to round, fluidal, white colonies with pink centers, in contrast to the common mutant which forms a round, butyrous, deep-red colony with a narrow, bluish border.

The Granada and Sequeira (1983) Modification to Improve Selectivity

Heat the TZC medium given above to 45° and add the following compounds per liter:

2, 3, 5-triphenyl tetrazolium chloride (as sterile 1% aqueous solution)	50 mg
Crystal violet	50 mg
Thimerosal (merthiolate)	5 mg
Polymyxin B sulfate	100 mg
Tyrosine	20 mg
Chloromycetin	5 mg
Cycloheximide (optional-to inhibit fungi)	50 mg
Chlorothalonil (optional-to inhibit fungi)	80 mg

The antibiotics and fungicides are sterilized by dissolving in 70% ethanol. The appearance of colonies is similar to TZC. Some strains of *P. solanacearum* are sensitive to chloromycetin and the concentrations need to be adjusted downward.

Other semi-selective media have been reported to be helpful for the following taxa: *P. glumae* (Tsushima et al., 1986), *P. pseudoalcaligenes* subsp. *konjaci* (Hayashi, 1987) *P. cepacia* (Burbage and Sassar, 1982), *P. cichorii* (Wu and

Thompson, 1984), and *P. s. pv. savastanoi* (Surico and Lavermicocca (1989). The CVP medium (Cuppels and Kelman, 1974; see also The Genus *Erwinia* in the second edition) also is helpful for identifying pectolytic pseudomonads such as *P. marginalis*.

## Identification of Phytopathogenic Pseudomonads

### Bacteriocin and Phage Relationships

Bacteriocins and phages have been useful in epidemiological studies and typing strains at the subspecific level. However, there are many problems associated with either technique because of absence of specificity. In addition, the techniques may be unreliable because of such factors as mutation of the host, mutation of the phage, and difficulty in defining host ranges (Okabe and Goto, 1963; Vidaver, 1976; Crosse and Garrett, 1963). The status and examples of research where phages and bacteriocins were used in the identification of various bacterial plant pathogens including *P. aeruginosa* were covered previously in *The Prokaryotes* (Schroth and Hildebrand, 1981). There has been little research in this area since then, primarily because of the development of more sophisticated techniques with greater powers of resolution. Methods using monoclonal antibodies, RFLP and genetic probes offer many advantages over past techniques. Epidemiological studies are frequently done with strains genetically marked for resistance to an antibiotic because of the ease with which large populations of a bacterial strain can be monitored.

### Genetic Marking of Bacterial Strains

The method of choice for monitoring the population dynamics of bacterial strains, whether they are pseudomonads or belong to other genera, is to genetically mark them for resistance to an antibiotic (Kloepper and Schroth, 1981; Bahme and Schroth, 1987). The most frequently used antibiotic is rifampicin because relatively few bacteria in nature are resistant to it. Also, stable resistant strains can be readily isolated whereas resistance and stability of the resistance often appears to be a problem with many other antibiotics such as nalidixic acid.

Caution must be exercised when using genetic marking such as rifampicin because it appears to affect the ecological competence of the strains (Lewis et al., 1987). We have noted in our investigations that strains marked with rifampicin may lose some of their properties such as reduced antibiotic production and a slower doubling time after a period of time. However, at present there

are no better alternatives that are available for field use when making numerous samplings.

### Serological Relationships

In the previous edition of *The Prokaryotes* (Schroth and Hildebrand, 1981), we detailed many examples where serology was used to discern relationships among the phytopathogenic pseudomonads. In general, serology complemented other techniques in confirming the identity of a strain. Serology was not widely used for identifying plant pathogenic bacteria because of lack of specificity of the antigenic components of the many *Pseudomonas* species and pathovars.

There have been great advances this past decade in the development of improved serological methods. The enzyme-linked immunosorbent technique (ELISA) and the specificity of monoclonal antibodies offer great potential for discerning strains and studying their epidemiology in nature. These new techniques have not yet been well exploited with the bacterial plant pathogens. However, there are some excellent examples of the usefulness of these techniques to identify strains with xanthomonads and fastidious bacterial plant pathogens (see Chapter 228).

### Fatty Acid Composition of Whole Cells

Sasser and his colleagues (Roy, 1988; Sasser, 1988; Sasser and Miller, 1984) have developed a sophisticated chromatographic method with computer analysis to identify many bacterial plant pathogens. The equipment and software is very expensive. The method is based on the finding that as many as 50 different fatty acids may be present in a cell. Bacterial cells are harvested from agar plates and suspended in methanol and sodium hydroxide in tubes which are sealed and heated. The esters are extracted in a suitable solvent, separated from the aqueous phase, and subsequently separated by gas chromatography. A library of profiles from known strains are part of the software which enable a comparison with unknown bacteria. The method appears to have good resolution for some pathovars (Roy, 1988). There are no publications available which clearly define the potential of this method for distinguishing the many pathovars and species of *Pseudomonas*.

## Preservation of Pseudomonad Cultures

Phytopathogenic and plant associated pseudomonads are typical bacteria that can be stored using conventional methods. Lyophilization

using skim milk or other menstrua works well. M. P. Starr successfully stored lyophils (skim milk as the menstruum) of pseudomonads for the International Collection of Phytopathogenic Bacteria (ICPB) for decades at room temperatures. Today, most investigators also freeze cultures at  $-70^{\circ}\text{C}$  or in liquid nitrogen ( $-196^{\circ}\text{C}$ ) for convenience. This works well for the phytopathogenic bacteria (Sly, 1983). Loss of viability is much less at the low temperatures. Commonly used cryoprotectants are 5–15% glycerol or dimethyl-sulfoxide (DMSO). We use 15% glycerol in our work. There are no doubt differences in the ability of various pseudomonads to survive at these temperatures and in lyophils. However, there is no ready source of information available to guide investigators.

Some investigators store bacteria in broth and water cultures and maintain them at room temperatures. In general this is done by placing about 5 loopfuls of bacteria in 10 ml of a rich broth or water (DeVay, 1963). We have maintained many cultures up to seven or more years using these techniques. However, the occurrence of mutations is a problem. Mutations are also inevitable when cultures are maintained by mass transfers. Our recommendation is to immediately freeze bacteria of interest to reduce the occurrence of mutations.

Mutations occur to some extent regardless of the storage method that is used. Although many strains may maintain characteristics of interest such as pathogenicity, there often are subtle changes which are not detected. These become evident when strains are tested in the field because any mutation is likely to reduce the ecological competence of an organism. This is a common problem when laboratory stored strains are reintroduced into their natural environments for ecological investigations.

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## *Xylophilus*

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### Introduction

*Xylophilus* is a genus of slow-growing, yellow-pigmented bacteria belonging to the family Comamonadaceae in the  $\beta$  subclass of the Proteobacteria. It comprises a single species, *Xylophilus ampelinus*, a phytopathogenic bacterium that, so far, has been isolated in different parts of the world only from diseased grapevines affected with bacterial necrosis and canker.

### Phylogeny

*Xylophilus ampelinus* was shown, initially by DNA:rRNA hybridizations (De Vos and De Ley, 1983; Willems et al., 1987) and more recently by 16S rDNA sequence analysis (Wen et al., 1999) to belong to the Comamonadaceae family in the  $\beta$  subclass of the Proteobacteria. Its nearest phylogenetic neighbors are the other members of this family: the genera *Acidovorax*, *Brachymonas*, *Comamonas*, *Delftia*, *Hydrogenophaga*, *Polaromonas*, *Rhodoferax* and *Variovorax*, and several genera of misnamed *Aquaspirillum* species  $\beta$  (Figure 1). Of all these taxa, *Variovorax paradoxus*, a yellow-pigmented hydrogen-oxidizing bacterium, appears to have a somewhat closer relationship with *Xylophilus ampelinus*. The 16S rDNA sequence similarity of these taxa is 97.9%, but the bootstrap value is 87% and therefore it is not clear if this relationship is significant (Willems and Gillis, in press).

EMBL Accession number of the 16S rRNA gene of *Xylophilus ampelinus* ATCC 33914 sequence is AF078758.

### Taxonomy

*Xylophilus ampelinus* was originally classified in the genus *Xanthomonas* because it is a Gram-negative, aerobic, nonsporeforming, rod-shaped, monotrichously flagellated, plant pathogenic bacterium that produces a yellow water-insoluble pigment and metabolizes sugars oxidatively (Panagopoulos, 1969). Later, several

reports (e.g., of pigment analysis); (Starr et al., 1977), comparative studies of the biosynthesis of aromatic amino acids (Byng et al., 1983; Whitaker et al., 1981), and DNA-rRNA hybridization studies (De Vos and De Ley, 1983) produced evidence showing that the grapevine pathogen was probably not a genuine *Xanthomonas* species (Bradbury, 1984). A polyphasic taxonomic study of more than 30 strains from different geographic origins by means of whole-cell protein analysis, numeric analysis of enzymatic features, DNA-DNA and DNA-rRNA hybridization, demonstrated that this organism indeed does not belong to *Xanthomonas*, but belongs in the so-called “acidovorans” rRNA complex, now known as the family Comamonadaceae in the  $\beta$  subclass of the Proteobacteria (Willems et al., 1991). Therefore the transfer of *Xanthomonas ampelina* to the new genus *Xylophilus* was proposed (Willems et al., 1987).

### Habitat

*Xylophilus ampelinus* strains have been isolated exclusively from different cultivars of *Vitis vinifera* displaying symptoms of bacterial necrosis and canker. Their occurrence has been reported from various vine-growing regions of the world, including the Mediterranean area, the Canary Islands, Austria, Switzerland, Bulgaria, South Africa and Argentina.

### Isolation

*Xylophilus ampelinus* can be isolated from grapevines affected by bacterial necrosis and canker. Isolation of strains may be complicated by the extremely slow and poor growth of *Xylophilus*, often permitting fast-growing saprophytes to overgrow isolation cultures (Panagopoulos, 1969).

Strains can be isolated throughout the year (Panagopoulos, 1969), although isolation from infected material collected in hot and dry periods may be difficult (Serfontein et al., 1997).

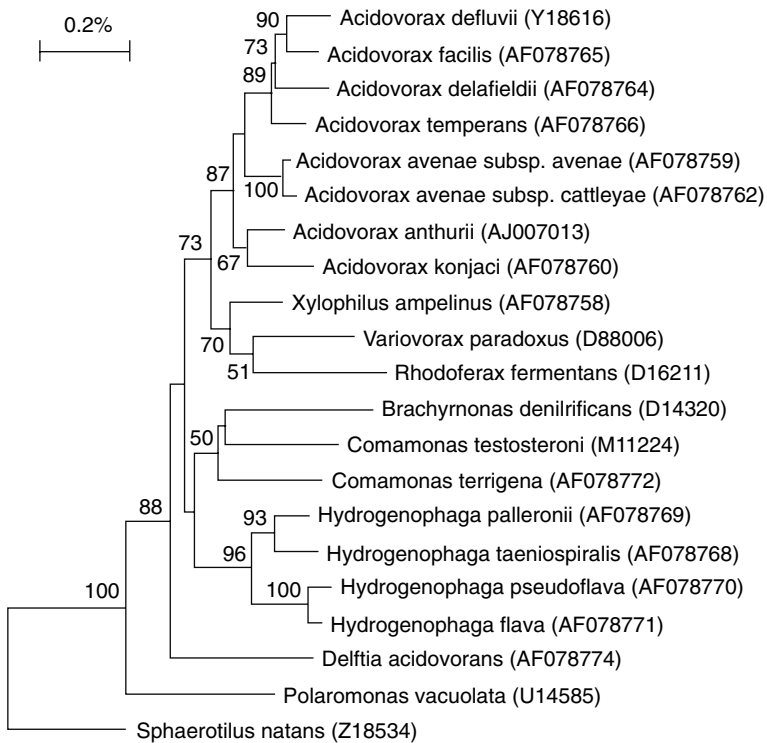


Fig. 1. Dendrogram obtained by neighbor-joining analysis of a distance matrix of 16S rDNA sequences, showing the position of *Xylophilus* among other members of the Comamonadaceae. Accession numbers are given in parentheses.

The most common isolation sources are small pieces of infected wood, taken aseptically from diseased vines and soaked for 20 minutes in sterile water. The resulting bacterial suspension is plated out onto nutrient agar (Panagopoulos, 1969; Erasmus et al., 1974; Grasso et al., 1979). After 5 to 6 days, small pale-yellow colonies will appear which, after 8 to 10 days, can attain a diameter of 0.4 to 0.6 mm (Panagopoulos, 1969). Isolation can be improved by incubating cuttings from diseased shoots that include a node, in closed plastic bags together with wet cotton plugs. After an incubation of 3 days in the dark at 15°C, extracts from the cuttings contained significantly more *Xylophilus ampelinus* bacteria than controls. This enrichment technique can also be applied to latently infected material without apparent signs of disease (Serfontein et al., 1997).

## Identification

Most characteristic for *Xylophilus ampelinus* strains is their extremely slow and poor growth on most media at the optimal growth temperature of 24°C, and these features allow their differentiation from other yellow-pigmented species of the Comamonadaceae. The following features permit differentiation from *Xanthomonas* species: very low salt tolerance, absence of

gas production from most sugars, failure to hydrolyze gelatin and esculin, positive urease reaction, growth on Na, K-tartrate and on L-glutamic acid, absence of growth on sucrose, and use of L-glutamic acid as sole carbon and nitrogen source (Van den Mooter and Swings, 1990).

Differentiation of *Xylophilus ampelinus* from other slow-growing organisms occurring on grapevine is possible by Gram reaction, catalase and oxidase tests, urease production and lipolysis of Tween 80 (Serfontein et al., 1997). In phytopathology laboratories, serological tests with specific antisera are used for rapid identification of the pathogen (Erasmus et al., 1974; Ridé, 1996). A polymerase chain reaction (PCR) identification test using the specificity of the 16S–23S rDNA spacer sequence was reported (Serfontein et al., 1997) but not published in detail. The EMBL accession number for the spacer sequence is U76357.

## Cultivation

### Media

*Xylophilus ampelinus* grows rather slowly on most media. In general, the use of a large inoculum is required for good growth. Media should be prepared freshly as *Xylophilus ampelinus* strains fail to grow on media that have been



reheated or sterilized twice (Panagopoulos, 1969).

Strains grow rather poorly on nutrient agar, but this may be improved by the addition of 5% sucrose. Best growth is reported on yeast extract-galactose-calcium carbonate (YGC) medium (composition [w/v] 1% yeast extract, 2% galactose, 2% CaCO<sub>3</sub>, 2% agar; Panagopoulos, 1969; Bradbury, 1973). Good growth is also obtained on glucose-yeast extract-calcium carbonate-agar (GYCA), containing (w/v) 0.5% yeast extract, 1% glucose, 3% CaCO<sub>3</sub> and 2% agar (Willems et al., 1987). The presence of CaCO<sub>3</sub> in both these media prevents the transmission of light, and therefore colony morphology is more conveniently studied on yeast extract-galactose (YEGAL) medium (composition in g liter<sup>-1</sup>: yeast extract, 5, galactose, 10, K<sub>2</sub> HPO<sub>4</sub>, 3.01, NaH<sub>2</sub> PO<sub>4</sub>, 4.55, NH<sub>4</sub>Cl, 1, MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5, ferric ammonium citrate, 0.05, CaCl<sub>2</sub>, 0.005, distilled water, 1 liter; yeast extract and galactose, each dissolved in 100 ml, should be autoclaved separately as concentrated solutions; Starr et al., 1977).

### Growth Conditions

Optimal growth temperature is 24°C, and minimum and maximum growth temperature are 6 and 30°C, respectively (Panagopoulos, 1969). On synthetic media, 0.1% glutamic acid is required as a growth factor (Panagopoulos, 1969; Bradbury, 1973).

### Colony Morphology

On nutrient agar, colonies are round with entire margins, slightly raised, semitranslucent, pale yellow and glistening; colony diameters are 0.2 to 0.3 mm after 6 days (0.6 to 0.8 mm after 15 days). Growth is better on GYCA medium where colonies are yellow. The yellow pigments of *Xylophilus* are sensitive to potassium isobutoxide but are very hard to purify and therefore not yet studied (Starr et al., 1977).

Occasionally *Xylophilus* strains produce two stable colony types, one type with relatively large yellow colonies (diameter 0.8 to 2.0 mm after 15 days on GYCA) and the other smaller, paler colonies (0.4 to 1 mm after 15 days on GYCA). Microscopically, as well as by analysis of total protein extracts by SDS-PAGE and DNA-DNA hybridizations, both types are usually highly similar (Willems et al., 1987).

### Preservation

Cultures that have been grown on screw-capped slants at 24°C for 2 to 3 days can be stored at 4°C,

caps tightly closed. They should be transferred at least every 2 months. For long-term preservation, strains can be lyophilized.

### Physiology

*Xylophilus ampelinus* strains have a strictly chemoorganotrophic metabolism. They use only a limited number of carbohydrates, organic acids and amino acids for growth. Of a total of 60 substrates tested, growth was recorded only on D-glucose, D-galactose, L-glutamic acid, Na-succinate, Na-fumarate, K, Na-tartrate, Na-L-malate, Na<sub>3</sub>-citrate and Ca-gluconate (Van den Mooter and Swings, 1990).

*Xylophilus* strains generally show very little variation. Even isolates originating from different geographical regions showed highly similar whole-cell protein SDS-PAGE patterns and little variation in enzymatic features, as tested with API ZYM systems (Willems et al., 1987). However, considerable variation between different populations was reported for the use of glucose and tartaric acid and for tyrosinase activity (Ridé, 1996).

A variation of the Biolog™ GN system in which the recommended sucrose peptone agar was replaced by nutrient agar, has been used to characterize several strains, but of the 95 substrates tested, only acetic acid, propionic acid, L-aspartic acid, L-glutamic acid and L-pyroglutamic acid were oxidized (Serfontein et al., 1997).

The regulation mechanisms of aromatic amino acid biosynthesis involves inhibition of 3-deoxy-D-arabinoheptulonate-7-phosphate synthetase by tryptophan, chorismate, prephenate, phenylalanine, and tyrosine. Co<sup>2+</sup> is needed for maximum activity. Prephenate dehydrogenase is NAD<sup>+</sup> specific and is not inhibited by tyrosine (Byng et al., 1983; Whitaker et al., 1981).

### Epidemiology

Recognition of the Causal Agent of Bacterial Necrosis of Grapevine *Xylophilus ampelinus* causes bacterial necrosis and canker of grapevines and is responsible for important economic losses to viticulture in several parts of the world. The disease was first described in France in 1895 as “Maladie d’Oléron” (Ravaz, 1895), but the actual causal agent was not isolated until 1969.

In 1939, “Tsilik marasi,” a disease of Cretan grapevines was reported and attributed to a fungus of the family Pythiaceae (Sarejanni, 1939). It was considered to be different from “Maladie d’Oléron” and “Gommose bacillaire” in France and from “Mal nero” in Italy. These three dis-

eases of grapevine and the similar “Vlamsiekte” in South Africa were at that time all attributed to *Erwinia vitivora* (Sarejanni, 1939; Du Plessis, 1940). Later this bacterium (syn. *Erwinia herbicola*, now *Pantoea agglomerans*) was shown to be an ordinary saprophyte, commonly isolated from infected plant material (Lelliott, 1974). In 1969, the isolation of a very slow growing, yellow-pigmented bacterium from grapevines with “Tsilik marasi” symptoms was reported. It was shown to be the causal agent of the grapevine disease and named *Xanthomonas ampelina* (Panagopoulos, 1969). It was then shown to be identical to the causal agent of “Maladie d’Oléron” (Prunier et al., 1970), “Vlamsiekte” (Erasmus et al., 1974) and “Mal nero” (Grasso et al., 1979).

### Geographical Distribution

*Xylophilus ampelinus* has been isolated from diseased grapevine in Greece, France, Sardinia, Sicily, Spain and South Africa (Panagopoulos, 1969; Prunier et al., 1970; Garau et al., 1987; Grasso et al., 1979; López et al., 1987; Erasmus et al., 1974). Similar disease symptoms were reported from Argentina, Austria, Bulgaria, the Canary Islands, Portugal, Switzerland, Tunisia, Turkey, the former Yugoslavia and the former USSR (Bradbury, 1984; Panagopoulos, 1987). The actual distribution of the disease may be much larger because its symptoms can easily be confused with those attributed to other bacteria such as *Pseudomonas syringae* and *Erwinia vitivora* (now *Pantoea agglomerans*; (Panagopoulos, 1969).

### Occurrence and Spreading of the Disease

Whereas bacterial necrosis and canker of grapevine was a rather rare disease at the beginning of the 20th century, it has since gained importance in the Mediterranean area through a combination of factors, including favorable local environmental conditions and agricultural practices, in particular increased mechanization of viticultural practices (Ridé, 1996). Inasmuch as *Xylophilus ampelinus* survives in the vascular tissue, transmission of the disease can easily occur via pruning lesions through the use of contaminated tools and infected grafting material. Wind and rain favor the spread of bacterial ooze from infected leaves, and severe winds and storms may cause wounds, thus providing additional access routes for the pathogen. Exceptionally dry seasons may result in the partial recovery of infected vineyards (Ridé, 1984; Panagopoulos, 1987; López et al., 1987).

### Pathogenicity

Bacterial necrosis and canker of grapevine caused by *Xylophilus ampelinus* first becomes apparent in early spring, when buds on affected shoots fail to open. Typical symptoms include longitudinal cracks and cankers that develop from hyperplasiae in the cambial tissue. The underlying vascular tissue shows a brown discoloration and eventually will die. Other parts of the shoot, less severely affected, may survive. Symptoms may develop on petioles, flower and fruit stalks, causing death of leaves, flowers or fruits. Leaves infected through hydathodes or stomata show reddish brown lesions. Roots may also be infected, resulting in retarded growth of shoots. The severity of symptoms may vary considerably on different grapevine varieties (Panagopoulos, 1969; Ridé, 1984; Grasso et al., 1979; López et al., 1987; Ridé, 1996).

There is no effective cure available and treatment consists of removing and burning infected shoots or, in cases of severe infection, removing and burning the whole plant. When pruning, tools should be disinfected before moving from one plant to the next. The only preventive measure that has given some success is treatment with Bordeaux mixture. In severely affected regions, the use of resistant grapevine cultivars is advisable (Ridé, 1996). An in vitro test to assess cultivar susceptibility involves inoculation of 2-month-old plants (having 8 to 12 internodes) by decapitation with scissors dipped in a bacterial suspension. The number of internodes showing disease symptoms after several weeks of incubation provides an estimate for the progression of infection (Peros et al., 1995).

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# The Genus *Acinetobacter*

KEVIN TOWNER

## Introduction

Gram-negative nonfermentative bacteria belonging to the genus *Acinetobacter* have been classified previously under at least 15 different names (Bergogne-Bérézin and Towner, 1996), and it is only relatively recently that rational taxonomic proposals have emerged. Delineation of species is still the subject of ongoing research, and a rapid and reliable method of assigning new isolates to individual species is particularly needed.

Notwithstanding the continuing taxonomic confusion, it is clear that acinetobacters are common, free-living saprophytes found in soil, water, sewage and foods. They are also ubiquitous organisms in the clinical environment, where they can be isolated as commensals from the skin of hospital staff and patients. They have increasingly been recognized as important nosocomial pathogens involved in outbreaks of hospital infection, particularly in high-dependency or intensive care units, where they rapidly develop resistance to even the most potent antimicrobials (Bergogne-Bérézin and Towner, 1996).

Although the significant pathogenic role of *Acinetobacter* is largely confined to nosocomial infections, it is important to note that these organisms are ubiquitous in both the clinical and nonclinical environments. Their metabolic versatility means that they may play an important role in a variety of commercially important industrial processes, as well as in the biodegradation of a wide range of environmental pollutants. While much remains to be learned about the lifestyles of the different members of the genus, it is clear that some facets of their behavior are unique and others provide new insights into prokaryotic behavior in general.

Members of the genus *Acinetobacter* have suffered a long history of extensive taxonomic change which has inhibited a proper appreciation and understanding of their significant biological properties and pathogenic importance. Although the delineation of species within the genus is still the subject of ongoing discussion and research, it appears that the current taxon-

omy should form a rational scientific foundation for the numerous important investigative studies required in the future.

## Phylogeny

Members of the genus *Acinetobacter* have historically been classified by various authors under a variety of different names (Bergogne-Bérézin and Towner, 1996) and consequently, much of the early literature concerning this group of organisms is difficult to interpret owing to confusion over phylogeny and the lack of a widely accepted classification scheme. The genus *Acinetobacter*, as originally proposed in 1954, included a heterogeneous collection of nonmotile Gram-negative saprophytes that could be distinguished from other similar bacteria by their lack of pigmentation. The *Subcommittee on the Taxonomy of Moraxella and Allied Bacteria* subsequently proposed in 1971 that the genus *Acinetobacter* should include only the oxidase-negative strains, and this division has been supported by the use of transformation tests (Juni, 1978) that still form a good basis for inclusion of isolates in the genus.

{*Bergey's Manual of Systematic Bacteriology*} classified the genus *Acinetobacter* in the family *Neisseriaceae* (Juni, 1984), but more recent molecular taxonomic studies have resulted in the proposal that *Acinetobacter* should be classified in the new family *Moraxellaceae* (Rossau et al., 1991). This new family includes *Acinetobacter*, *Moraxella*, *Psychrobacter* and related organisms, and constitutes a discrete phylometric branch in superfamily II of the Proteobacteria on the basis of 16S rRNA studies and rDNA-DNA hybridization assays (van Landschoot et al., 1986; Rossau et al., 1989).

## Taxonomy

Delineation of species within the genus *Acinetobacter* is still the subject of much research. Phenotypic identification of individual species is

complex and time-consuming (Gerner-Smidt et al., 1991). However, using the formal molecular definition (Wayne et al., 1987) of a microbial species — i.e., that a species should include strains of approximately 70% or greater DNA-DNA relatedness and 5°C or less divergence values ( $\Delta T^m$ ) — more than 20 separate genomic species (DNA-DNA homology groups) have been recognized within the genus by different research groups on the basis of DNA hybridization studies. Based on the taxonomic recommendation that only genomic groups readily distinguishable by phenotypic methods and containing more than 10 strains should be given names, seven *Acinetobacter* genomic species have been given formal species names (Table 1). Genomic species 1, 2, 3 and 13TU are closely related and are sometimes referred to as the *A. calcoaceticus*–*A. baumannii* complex for epidemiological purposes.

Certain genomic species were described independently by Bouvet and Jeanjean (1989) and Tjernberg and Ursing (1989a), and there are some minor discrepancies in the numbering systems. To avoid further confusion, it is current practice to add the suffix BJ or TU to denote the genomic species delineated by the two studies. In addition, many reports in the scientific literature have reported *Acinetobacter* isolates that cannot be identified with any of the formally recognized genomic species. One such isolate from the Venice lagoon has been given the species name *A. venetianus* by its discoverers Di Cello et al. (1997) and has been shown to belong to the same

genomic species as the industrially important strain RAG-1 (Vanechoutte et al., 1999). Because many of the strains studied in DNA-DNA hybridization studies have been derived from hospital sources, and because the most common habitats of these organisms are soil and water, it seems clear that many naturally occurring genomic species of *Acinetobacter* have yet to be delineated and that the current taxonomic listing is incomplete (Nemec et al., 2000). Unfortunately, many reports of *Acinetobacter* in the scientific and medical literature still do not use the latest taxonomy or use inadequate identification methods. Although phenotypic identification is problematical, various molecular methods have been developed in an attempt to provide a rapid identification method suitable for routine taxonomic and epidemiological use.

## Habitat

Certain acinetobacters are normal inhabitants of human skin, and members of the genus have increasingly been implicated as a presumed causal or contributory agent in numerous infectious disease processes. In addition to their occurrence in a range of clinical specimens (e.g., tracheal aspirates, blood and urine), acinetobacters can be readily isolated from moist skin areas, such as toe webs, the groin and the axilla. Other reservoirs of these organisms may include a range of both moist and dry surfaces and equipment within the hospital environment as well as

Table 1. Formally recognized genomic species of *Acinetobacter*.<sup>a</sup>

Genomic species number	Genomic species name	Type strain
1	<i>Acinetobacter calcoaceticus</i>	ATCC 23055
2	<i>Acinetobacter baumannii</i>	CIP 70.34
3	Not named	ATCC 19004
13TU	Not named	ATCC 17903
4	<i>Acinetobacter haemolyticus</i>	ATCC 17906
5	<i>Acinetobacter junii</i>	ATCC 17908
6	Not named	ATCC 17979
7	<i>Acinetobacter johnsonii</i>	ATCC 17909
8	<i>Acinetobacter lwoffii</i>	ATCC 15309
9	Not named	ATCC 9957
10	Not named	ATCC 17924
11	Not named	ATCC 11171
12	<i>Acinetobacter radioresistens</i>	IAM 13186
13BJ	Not named	ATCC 17905
14	Not named	Bouvet 382
15BJ	Not named	Bouvet 240
15TU	Not named	Tjernberg 151a
16	Not named	ATCC 17988
17	Not named	Bouvet 942

<sup>a</sup>Numerous published reports refer to *Acinetobacter* isolates that cannot be identified with any of the genomic species listed above. Such new isolates have not yet been formally grouped or given species names, although a newly named species, *Acinetobacter venetianus*, has been proposed (Di Cello et al., 1997).

the patients and staff (Bergogne-Bérézín and Towner, 1996).

Many studies within the genus *Acinetobacter* have been performed with clinical isolates. However, acinetobacters are also ubiquitous organisms in soil, water and sewage (Towner, 1996). It has been estimated that *Acinetobacter* may constitute as much as 0.001% of the total heterotrophic aerobic population of soil and water (Baumann, 1968). They have been found at densities exceeding  $10^4$  organisms per 100 ml in freshwater ecosystems and  $10^6$  organisms per 100 ml in raw sewage (LaCroix and Cabelli, 1982). They can be isolated from heavily polluted water, such as that found in wastewater treatment plants, but are found more frequently near the surface of fresh water and where fresh water flows into the sea (Droop and Jannasch, 1977).

Acinetobacters also are found in a variety of foodstuffs, including eviscerated chicken carcasses, various poultry and other meats, milk products and vegetables. It has been reported that acinetobacters constitute up to 22.7% of the total microflora of chicken carcasses. It is also known that acinetobacters are involved in the economically important spoilage of foods such as bacon, chicken, eggs and fish, even when stored under refrigerated conditions or following irradiation treatment (Towner, 1996).

It is worth noting that there is a significant population difference between the acinetobacters found in clinical and other environments. The vast majority of clinically significant isolates belong to the *A. calcoaceticus*-*A. baumannii* complex, whereas genomic species 7 (*A. johnsonii*), 8 (*A. lwoffii*) and 9 seem to predominate in foods and the environment. Other genomic species appear to comprise only minority components of the different populations investigated, but they may have evolved to acquire a selective advantage in as yet unrecognized specialized ecological niches.

## Isolation

Isolation of members of the genus *Acinetobacter* can be accomplished using standard laboratory media such as trypticase soy agar or brain heart infusion agar. A differential medium such as MacConkey agar may be helpful in recognizing colonies of *Acinetobacter* on primary isolation. The optimum growth temperature for most strains is 33–35°C, but many clinically significant isolates will grow well at 37–42°C. In contrast, some environmental and food spoilage isolates have considerably lower optimum growth temperatures and may be unable to grow at 37°C.

## Selective Enrichment

Most strains of *Acinetobacter* can grow in a simple mineral medium containing a single carbon and energy source such as acetate, lactate or pyruvate. An enrichment culture procedure for isolating members of the genus from soil and water has been described (Baumann, 1968). Liquid enrichment cultures containing 20 ml of medium (see below) are inoculated with a 5-ml sample of water, or a filtered 10% soil suspension, and vigorously aerated at either 30°C or room temperature. Cultures are examined microscopically after 24 or 48 h and streaked on to suitable isolation media. Strains of *Acinetobacter* have a slightly acid pH optimum for growth, and vigorous aeration at a pH of 5.5–6.0 favors their enrichment.

### Baumann's Enrichment Medium

Sodium acetate (trihydrate)	2 g
KNO <sub>3</sub>	2 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.2 g

Dissolve in 0.04 M KH<sub>2</sub>PO<sub>4</sub>.Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.0), to a total volume of 1 liter.

It should be noted that the original recipe (Baumann, 1968) also contained a complex trace element supplement, but this addition is now rarely considered to be necessary as there are sufficient trace elements in the other medium components.

## Differential Isolation on Solid Medium

Selective liquid enrichment is rarely used for isolation of *Acinetobacter* from clinical specimens. For clinical isolation, general purpose media such as blood agar or MacConkey agar are usually preferred because of their broad bacterial coverage, but in certain circumstances, it may be preferable to use a selective medium that suppresses the growth of other bacteria. Such a medium also can be used for plating out liquid enrichment cultures. An antibiotic-containing selective medium, Leeds *Acinetobacter* Medium (see below), has been found useful for the recovery of most *Acinetobacter* genomic species from clinical and environmental sources (Jawad et al., 1994).

### Leeds *Acinetobacter* Medium

Bacteriological agar	15 g
Acid casein hydrolysate	15 g
Neutralized soy peptone	5 g
NaCl	5 g
D-(–) Fructose	5 g
Sucrose	5 g
D-Mannitol	5 g
L-Phenylalanine	1 g
Ferric ammonium citrate	0.4 g
Phenol red	0.02 g

Add ingredients to 1 liter of distilled water. Steam to dissolve ingredients. Mix well. Adjust pH of medium to 7.0. Autoclave for 15 min at 121°C and 15 lb/in<sup>2</sup>. Cool to 50–55°C and then add the following ingredients (per liter): 10 mg of vancomycin, 15 mg of cefsulodin and 50 mg of cephadrine.

Mix well. Pour on plates. Pack in plastic bags and store at 4°C for up to 2 weeks.

## Identification

### Morphology

Short, plump, Gram-negative rods, typically 0.9–1.6 by 1.5–2.5 µm in the logarithmic phase of growth, but often becoming more coccoid in the stationary phase. The cell wall ultrastructure is typical of Gram-negative bacteria in general, but the cells are occasionally difficult to destain. Cells commonly occur in pairs, but also in chains of variable length. No spores are formed and flagellae are absent. Although generally considered to be nonmotile, “twitching” or “gliding” motility has been reported to occur, particularly on semisolid media. Many strains are encapsulated, and the capsule may be readily seen in India wet ink mounts. Colonies are usually nonpigmented, but some strains form white-to cream-colored colonies, which vary in consistency from butyrous to smooth and mucoid, and from 1–2 mm in diameter.

### Phenotypic Identification

All members of the genus *Acinetobacter* are strict aerobes and can grow at a wide range of temperatures. Most strains will grow at 33°C, but some environmental isolates prefer incubation temperatures from 20–30°C. Clinical isolates of *Acinetobacter* will normally grow at 37°C and some strains can grow at 42°C. All acinetobacters are oxidase-negative and catalase-positive. The negative oxidase reaction serves to distinguish the genus from other related genera. Most strains of *Acinetobacter* can grow in a simple mineral medium containing a single carbon and energy source. A wide variety of organic compounds can be used as carbon sources by particular strains, although relatively few strains can use glucose. Most strains are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay. A few clinical strains may show hemolysis on blood agar plates owing to the production of phospholipase C. Unlike other members of the *Moraxellaceae*, most strains are resistant to penicillin and many clinical isolates are resistant to cephalosporin antibiotics because of the overproduction of a chromosomal cephalosporinase.

There is no single biochemical test that enables ready differentiation of this genus from similar bacteria, but the nonfastidious nature and wide biochemical activities of the members of the genus makes them readily distinguishable from other bacteria at the genus level by the combination of nutritional tests applied to nonfastidious, nonfermentative organisms in general, including most commercially available diagnostic devices and systems. Phenotypic identification to the genomic species level is more problematic and time-consuming. A scheme of 22 phenotypic tests has been described that differentiates most of the genomic species known at the present time (Kämpfer et al., 1993), but this scheme is laborious and time-consuming. Most commercial rapid identification systems are inadequate for distinguishing the different genomic species, but promising results have been obtained with the automated Biolog system which involves the detection of oxidation with 95 different carbon sources (Dijkshoorn, 1996).

### Serological Identification

A large number of capsular types have been identified by means of capsular reactions or immunofluorescence. This has resulted in the delineation of a large number of different serovars in two of the main genomic species (spp. 2 and 3) associated with infection in man (Traub and Leonhard, 1994). Inasmuch as all recently investigated lipopolysaccharide (LPS) molecules from clinical *Acinetobacter* isolates have been shown to be of the smooth phenotype, a serotyping scheme for identification of clinically important members of the genus may be possible (Pantophlet et al., 1998).

### Genetic Identification

Members of the genus are themselves only rarely transformable, but can be easily identified by testing the ability of their isolated DNA to transform a nutritional or antibiotic resistance marker to the naturally competent strain BD413 originally isolated by Juni (1972). Transformation of this highly competent strain occurs readily, either on semisolid media or in liquid culture, and has been used as the basis of a test for the identification of *Acinetobacter* in clinical specimens (Brooks and Sodeman, 1974). The DNA samples from unrelated bacteria have consistently failed to transform the auxotrophic strain BD413 to prototrophy (Juni, 1972), and this test is consequently believed to allow unambiguous attribution of unknown strains to the *Acinetobacter* genus. There is currently no genetic test for the identification of individual genomic species.

## Molecular Identification

A genus-specific 16S rDNA-targeted oligonucleotide probe has been used to recognize acinetobacters in general (Wagner et al., 1994). However, most work on the development of molecular methods has been dedicated to developing methods for distinguishing the individual genomic species. The "gold standard" method is DNA-DNA hybridization (Tjernberg et al., 1989b), but this technique is rather laborious and is normally used only in special situations in reference laboratories. Consequently, many research groups have concentrated on the development of alternative molecular methods for distinguishing individual genomic species. Unambiguous differences in rDNA sequences have been found in the highly variable regions of 16S rDNA molecules from at least 21 different genomic groups (Ibrahim et al., 1997), although the limited number of strains examined means that these findings cannot be relied upon for absolute identification of genomic species at the present time. It also should be noted that the groupings based on 16S rDNA analysis did not completely correlate with those based on DNA-DNA homology data. This is in contrast with an alternative strategy in which phylogenetic groupings were based on the nucleotide sequences of topoisomerase (*gyrB*) genes (Yamamoto and Harayama, 1996).

As an alternative to direct sequence-based identification, a range of more rapid molecular fingerprinting methods have been developed for distinguishing individual genomic species, with varying degrees of success. These methods can be divided into those based on structural features, such as outer-membrane protein patterns (Ino and Nishimura, 1989; Dijkshoorn et al., 1990), and those based on nucleic acid analysis. The most widely used techniques amongst the latter group include amplified fragment length polymorphism (AFLP) analysis (Janssen and Dijkshoorn, 1996), amplified rDNA restriction analysis (ARDRA; Vaneechoutte et al., 1995; Dijkshoorn et al., 1998), ribotyping (Gerner-Smidt, 1992), tDNA spacer fingerprinting (Ehrenstein et al., 1996) and 16S-23S spacer analysis (Dolzani et al., 1995).

## Preservation

Cultures of *Acinetobacter* spp. can be stored for short periods (a few weeks) on nutrient agar slants kept at room temperature. It should be noted that many strains do not seem to survive for more than short periods in the refrigerator at 4°C. For long-term preservation, lyophilization should be used, or a heavy suspension of cells in brain heart infusion broth supplemented with

20% v/v glycerol can be frozen at -80°C. For regrowth, it is usually possible to simply streak out a loopful of the frozen stock without the necessity to thaw the entire suspension.

## Physiology

The main identifying biochemical and physiological characteristics of the genus *Acinetobacter* have been discussed in the context of identification and only the most significant aspects are reiterated in this section. Early work on the metabolism of the genus has been comprehensively reviewed previously (Juni, 1978). Numerous subsequent papers describing enzymes, cofactors, metabolic pathways, products and other physiological aspects have confirmed that the *Acinetobacter* are strictly aerobic and broadly typical of other Gram-negative eubacteria, but with a number of distinctive physiological features that support the versatile lifestyle of this genus. It is worth emphasizing that many strains of *Acinetobacter* used in physiological studies were originally isolated in the 1970s or earlier and have never been properly identified to the genomic species level, and that many detailed physiological studies have been based on work with only one or a very limited number of strains.

Although rare strains of *Acinetobacter* showing growth factor requirements have been isolated, the vast majority of strains resemble saprophytic pseudomonads in being able to use any of a large range of organic compounds as a carbon and energy source in an otherwise mineral medium. Although the utilization of carbohydrates is relatively uncommon, the major biochemical feature of the genus is that many strains are able to metabolize a range of compounds including aliphatic alcohols, some amino acids, decarboxylic and fatty acids, unbranched hydrocarbons, sugars, and many relatively recalcitrant aromatic compounds such as benzoate, mandelate, *n*-hexadecane, cyclohexanol and 2,3-butanediol (Juni, 1978). Many such aromatic compounds are converted by acinetobacters to  $\beta$ -ketoacid, which is degraded in turn to succinate and acetyl-CoA (it should, however, be noted that the precise spectrum of compounds degraded is frequently strain-specific). Members of the genus are therefore particularly suitable organisms for studying a variety of unusual biochemical pathways, and they also may have a role to play in degrading a range of pollutants and industrial products.

Central metabolism seems to be based on a Krebs tricarboxylic acid cycle, but with a rather unusual regulatory system in which AMP simultaneously modulates the activities of a number



of the constituent enzymes. This feature may act in a concerted manner to direct metabolic flux through the cycle, and such multipoint regulatory control may be a significant feature of energy metabolism in *Acinetobacter* spp. Numerous complex catabolic pathways feed into the tricarboxylic acid cycle, and various biosynthetic pathways, such as those involved in gluconeogenesis and amino acid synthesis, are subtly influenced by the need to integrate with the overall metabolism of the organism (Towner et al., 1991b). Most strains are unable to utilize glucose as a carbon source, but occasional rare strains are able to do so via the Entner-Doudoroff pathway. Many acinetobacters are, however, able to acidify media containing sugars, including glucose, via an aldose dehydrogenase. This property has previously been considered to be of major taxonomic significance in the subdivision of the genus, but DNA-DNA hybridization studies now suggest that this is not the case unless considered in combination with unrelated biochemical properties. Nevertheless, the property of glucose acidification is still sometimes useful for initial presumptive identification of the main genomic species (the *A. calcoaceticus*-*A. baumannii* complex) involved in human disease.

Although most strains of *Acinetobacter* are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay, both nitrate and nitrite can be used as nitrogen sources by means of an assimilatory nitrate reductase. All acinetobacters are oxidase-negative, since they lack cytochrome *c*, but they do contain cytochromes *b*, *o* and occasionally *d* and *P-450* cytochrome.

One important difference from many organisms is that *Acinetobacter* cannot incorporate extracellular thymine or thymidine into DNA. Enzyme analysis has revealed that *Acinetobacter* lacks the enzymes thymidine phosphorylase, nucleoside deoxyribosyltransferase and thymidine kinase, but does contain enzymes for conversion of thymidine-5'-monophosphate to thymidine-5'-triphosphate. Polyphosphate has been shown to be accumulated by some acinetobacters in wastewater, and it has been suggested that these organisms could possibly remove inorganic phosphate from sewage in activated sludge. A high-affinity phosphate uptake system, inducible in the absence of inorganic phosphate (Kortstee et al., 1994), and a polyphosphate kinase induced by phosphate starvation (Geissdörfer et al., 1998) have both been demonstrated.

For further information on these and other biochemical pathways found in *Acinetobacter*, the interested reader should consult detailed reviews available elsewhere (Juni, 1978; Towner et al., 1991b; Towner et al., 1996).

## Genetics

### Modes of Gene Transfer

All three of the major modes of gene transfer are known to occur in *Acinetobacter*.

### Transformation

The transfer of genetic material by transformation in a strain of *Acinetobacter* was first demonstrated in 1969 and forms the basis of the genetic test for the identification of members of the genus (Juni, 1972). Genetic competence for transformation seems to be a rare trait in *Acinetobacter* (Juni, 1978), and most transformation studies have been performed with the highly competent strain BD413. Studies of the conditions for quantitative transformation in strain BD413 have shown that competence occurs throughout the life cycle, but with a peak early in the exponential growth phase (Cruze et al., 1979). The highest transformation frequencies (0.5–0.7%) were obtained in an aspartate-containing medium which allowed the most rapid growth of BD413. Recipient cell concentrations of  $1-6 \times 10^6$  cells per ml were found to give the highest transformation frequencies, regardless of the DNA concentration. Similar findings were reported with strain NCIB 8250 (Ahlquist et al., 1980), which seemed to have two peaks of competence, one during the early phase of batch culture and a second minor peak at the beginning of the stationary phase. The presence of cyclic AMP was found to increase the transformation frequencies obtained. Investigations into the basis for DNA uptake in competent strains of *Acinetobacter* have associated DNA uptake with components involved in assembly of the type IV pilus (Palmen and Hellingwerf, 1997; Link et al., 1998).

Nutritional and other markers (e.g., antibiotic resistance) are all transformed readily to competent strains. Early studies in the 1970s used transformation for preliminary genetic mapping of genes concerned with capsule biosynthesis, tryptophan biosynthesis and proline biosynthesis. More recently, catabolic pathways in *Acinetobacter* have been analyzed by natural transformation (Kloos et al., 1995), and the process has also been used to introduce randomly mutated genes into the chromosome of a competent recipient strain (Kok et al., 1997). Using previously isolated *Acinetobacter* genes modified to incorporate a *NotI* recognition sequence, the process of natural transformation has been exploited to generate a physical and genetic map of more than 40 genes on the circular c.3.8-kb chromosome of strain ADP1 (BD413UE; Galton et al., 1997).

## Transduction

A large number of bacteriophages active against specific strains of *Acinetobacter* have been isolated (Ackermann et al., 1994). Most *Acinetobacter* phages are lytic, but one temperate phage (P78) which lysogenizes its host strain and is capable of mediating generalized transduction has been isolated (Herman and Juni, 1974). Phage P78 is specific for its host strain and failed to lysogenize 389 other independently isolated strains of *Acinetobacter*, including the transformation-competent strain BD413. This narrow host specificity may be accounted for by the large number of different surface antigens found in this genus (Marcus et al., 1969). At present, neither P78 nor any other bacteriophage has been used for extensive genetic studies in *Acinetobacter*.

## Conjugation

Conjugation in the genus *Acinetobacter* was first reported in 1976 in strain EBF65/65 (Towner and Vivian, 1976a). The broad-host-range plasmid RP4 was used as a vector and was found to be capable of mobilizing chromosomal genes between different mutant derivatives of EBF65/65. Transfer of the chromosome occurred at detectable frequencies only on solid surfaces (not in liquid matings) and mobilization occurred from at least two different chromosomal locations (Towner and Vivian, 1976b). Chromosomal transfer also has been shown to be mediated by the naturally occurring *Acinetobacter* plasmid pAV1 (Hinchliffe and Vivian, 1980). Little is known about the precise nature and events involved in mobilization of the chromosome by RP4 and pAV1, but these systems have been successfully used to map a number of different mutations on a circular chromosomal linkage group in strain EBF65/65 (Towner, 1978; Vivian, 1981; Vakeria et al., 1984). A range of plasmids belonging to different incompatibility groups are capable of transfer by conjugation to *Acinetobacter* from enteric bacteria, although not all are stably maintained or capable of subsequent rounds of transfer (Chopade et al., 1985).

## Genetic Organization and Regulation

So far as the gross topological structure of the chromosome is concerned, it is known only that the chromosome of strains EBF65/65 and BD413 is circular. There is some preliminary evidence that *Acinetobacter* is more akin to *Pseudomonas* than enteric bacteria in that functionally related genes can be located at several different positions on the chromosome (Towner, 1978; Gralton et al., 1997).

In contrast, knowledge of the fine structure of certain specific regions of the chromosome is quite well advanced for the highly competent *Acinetobacter* strain BD413. Particular attention has focused on the organization and regulation of genes concerned with the tryptophan biosynthetic and the  $\beta$ -keto adipate degradative pathways. Striking similarities exist between the individual genes from *Acinetobacter* spp. and *Pseudomonas* spp. in these pathways, but considerable gene rearrangements seem to have occurred during the evolutionary process. Although the complete regulatory circuits for the tryptophan biosynthetic pathway have yet to be fully resolved in *Acinetobacter*, studies of the  $\beta$ -keto adipate pathway have indicated that *Acinetobacter* strain BD4 exhibits induction patterns unlike those found in *Pseudomonas* spp. However, an important characteristic shared with *Pseudomonas* is that genes for physiologically interdependent steps in the  $\beta$ -keto adipate degradative pathway tend to be linked together in supra-operonic clusters, a feature that may have important regulatory implications. Mapping and regulatory studies on these and related pathways in strain BD413 continue to be published regularly in the scientific literature. Detailed information on the formative studies can be found in previously published review articles (Haspel et al., 1991; Ornston and Neidle, 1991).

## Plasmids

Several studies have reported that more than 80% of *Acinetobacter* isolates carry multiple indigenous plasmids of variable molecular size (Gerner-Smidt et al., 1989; Seifert et al., 1994), although other studies have reported problems in isolating plasmid DNA from *Acinetobacter* spp., often because of unappreciated difficulties in lysing the cell wall of these organisms. Most indigenous plasmids from acinetobacters seem to be relatively small (<23 kb), and therefore probably lack conjugative functions.

As with many other groups of organisms, interest has focused particularly on plasmids associated with resistance to antibiotics. Although many clinical isolates of *Acinetobacter* show widespread and increasing resistance to a whole range of antibiotics, few studies have demonstrated plasmid-mediated transfer of resistance genes. This may partly reflect a lack of conjugative functions on indigenous plasmids, but also may reflect the absence of a suitable test system for detecting such transfer. For historical reasons, attempts to transfer plasmids from clinical isolates of any Gram-negative species have tended to use *Escherichia coli* K12 as a recipient strain. Complex and varied transfer frequencies

of standard plasmids belonging to different incompatibility groups have been observed between *E. coli* K12 and *Acinetobacter* strain EBF 65/65, and a number of these plasmids required an additional mobilizing plasmid for retransfer to occur (Chopade et al., 1985). Accordingly, it is not surprising that most reported cases of indigenous transmissible antibiotic resistance from *Acinetobacter* have been associated with plasmids belonging to broad host-range incompatibility groups (Towner, 1991a).

Apart from antibiotic resistance, genes encoding resistance to heavy metals (Kholodii et al., 1993) and important metabolic steps in the degradation of organic compounds and environmental pollutants, such as polychlorinated biphenyls (PCBs), have been shown to be carried on plasmids in *Acinetobacter* (Towner, 1991a; Fujii et al., 1997). Studies to date indicate clearly that though there is a pool of plasmid-mediated genetic information that is confined largely to *Acinetobacter*, a group of plasmids can cross the boundaries between *Acinetobacter* and other distinct genetic pools.

A range of cloning and shuttle vectors for in-vitro genetic manipulation experiments in *Acinetobacter* have been described (Ditta et al., 1985; Singer et al., 1986; Hunger et al., 1990; Gutnick et al., 1991; Minas and Gutnick, 1993).

### Transposons and Integrons

Transposons probably play an important role in ensuring that particular novel genes can become established in a new gene pool, even if the plasmid vectors that transferred them are unstable. There have been several reports of chromosomally located transposons carrying multiple antibiotic resistance genes in clinical isolates of *Acinetobacter* (Towner, 1991a). In general, such transposons closely resemble those found in other Gram-negative bacteria. Transposons also have been used in conjunction with suicide plasmid vectors to introduce mutations to the *Acinetobacter* chromosome (Towner, 1991a; Leahy et al., 1993).

Integrons are conserved genetic elements which encode a site-specific recombination system that enables the insertion, deletion and rearrangement of discrete genetic cassettes within the integron structure (Stokes and Hall, 1989). Most, but not all, cassettes identified to date have been associated with antibiotic resistance, and large numbers of clinical isolates of *Acinetobacter* have been shown to carry integrons incorporated into their chromosome (Gonzalez et al., 1998; Seward and Towner, 1999; Gallego and Towner, 2001). It is clear that clinical isolates of *Acinetobacter* seem to share resis-

tance mechanisms with many other genera, and it has been suggested that integron structures make an important contribution to the dissemination of antibiotic resistance genes in the clinical setting.

## Ecology

### Species of Clinical Importance

In addition to carriage by patients and staff, various strains of *Acinetobacter* can be isolated from a wide range of both moist and dry inanimate sources in the hospital environment, including formica table tops, dust, ventilatory equipment, humidifiers, mattresses, urinals and wash basins. Numerous studies have now supported the observation that *A. baumannii* and its close relatives are the main genomic species associated with outbreaks of hospital infection. Thus, a typical study of 584 *Acinetobacter* isolates from 420 patients at 12 different hospitals over a 12-month period identified 426 (72.9%) strains as *A. baumannii* (Seifert et al., 1993). This large study also identified 158 isolates as belonging to species other than *A. baumannii*, of which the most common were *Acinetobacter* sp. 3 (55 isolates), *A. johnsonii* (29 isolates) and *A. lwoffii* (21 isolates).

Very little is known about the clinical significance of other *Acinetobacter* genomic species and further detailed investigations are required. The ubiquitous occurrence of acinetobacters in the environment, and as commensals on human skin, means that such isolates in clinical specimens are often considered to be contaminants. Diagnosis of infection with "unusual" *Acinetobacter* genomic species therefore often depends on clinical indications and repeated isolation of the same strain from a single patient. *Acinetobacter* spp. 3 and 13TU have often been implicated in documented outbreaks of infection involving nosocomial spread, whereas *A. junii* and *A. johnsonii* have been associated with point-source outbreaks. It is worth re-emphasising the close relationship between genomic species 1, 2, 3 and 13TU. This "*A. calcoaceticus*-*A. baumannii* complex" contains isolates that are mostly glucose-acidifying. The majority of glucose-negative, nonhemolytic strains found in clinical specimens seem to be identified mainly as *A. lwoffii*, *A. johnsonii* or *Acinetobacter* sp. 12, and it seems that these species are natural inhabitants of human skin. Most of the hemolytic isolates are identified as *A. haemolyticus* or *Acinetobacter* sp. 6. Other groups seem to be implicated only occasionally in human infections (Bergogne-Bérézin and Towner, 1996).

## Species from the Environment

Acinetobacters are ubiquitous organisms that can be obtained easily from soil, food, water and sewage with appropriate enrichment techniques, but surprisingly few studies exist in which *Acinetobacter* isolates from environmental sources have been grouped according to the latest taxonomic criteria.

So far as food isolates are concerned, a study of 170 *Acinetobacter* isolates from various food sources (including fresh and spoiled meat and fish, vegetables, raw milk and cheese) demonstrated a clear difference in the distribution of genomic species between food and clinical isolates (Gennari and Lombardi, 1993), with *A. lwoffii* and *A. johnsonii* predominating in foods. The ubiquitous occurrence of *A. johnsonii* in the environment has also been demonstrated in several studies of polyphosphate-accumulating acinetobacters found in wastewater treatment plants (Kämpfer et al., 1992; Knight et al., 1993).

It therefore appears that there is a significant population difference between the acinetobacters found in clinical and other environments. Each population appears to be characterized by predominant groups of genomic species — spp. 2 (*A. baumannii*) and 3 in clinical environments, and spp. 7 (*A. johnsonii*) and 8/9 (*A. lwoffii*) in other environments. Other genomic species appear to constitute only minority components of the different populations. Some genetic interchange between populations is clearly possible for those groups that are capable of growing at both 37°C and lower environmental temperatures, and this fact may have implications for the spread of antibiotic resistance and metabolic genes.

## Epidemiology

### Human Carriage

At least 25% of healthy individuals may carry acinetobacters as part of their normal skin flora, but carriage of *Acinetobacter* spp. by healthy subjects at other body sites is normally low (Bergogne-Bérézin and Towner, 1996). In contrast, high colonization rates of the skin, throat, respiratory tract or digestive tract of hospitalized patients with clinically significant strains have been reported during outbreaks of infection, with infections involving mechanically ventilated intensive care unit (ICU) patients being associated particularly with a high colonization rate of the respiratory tract. Such patients often have heavy skin colonization, which probably contributes to the spread and persistence of outbreaks via the hands of hospital staff during trivial contacts (Bergogne-Bérézin and Towner, 1996).

From a clinical viewpoint, the isolation of strains belonging to genomic species 1, 2, 3 or 13TU in environmental cross-infection studies should be considered to be of greater epidemiological significance than the detection of genomic species associated mostly with nonhuman sources. The observed discrepancies in carriage rates between hospitalized patients and the normal population suggest that infecting or colonizing organisms in hospitals may be derived more often from cross-transmission or hospital environmental sources than from endogenous sources (e.g., the intestinal and digestive tract of the patients themselves). Differentiation of colonization from infection is not always easy, and many isolates, particularly from the skin, should be considered as colonizing rather than infecting organisms. However, the skin, respiratory tract and superficial wounds also should be considered as potentially important reservoirs of infecting organisms during outbreak situations (Bergogne-Bérézin and Towner, 1996).

### Environmental Persistence

*Acinetobacter* spp. spread easily in the environment of infected or colonized patients, and can persist in that environment for many days or weeks, even in dry conditions on particles and dust. Air contamination is relatively rare, except in the immediate vicinity of infected or colonized patients, but numerous studies have documented the isolation of acinetobacters from various hospital equipment and surfaces (e.g., respirators, air humidifiers, mattresses, pillows, bedside cabinets and cupboards, telephones, door handles, patient charts, floors, washcloths, etc.; Bergogne-Bérézin and Towner, 1996). Survival in the environment is probably helped by the ability of *Acinetobacter* spp. to resist drying (Jawad et al., 1996) and to grow at a range of different temperatures and pH values (Bergogne-Bérézin and Towner, 1996). The population differences found between “clinically significant” and “environmental” isolates means that it is important to identify isolates to the genomic species level and type them (Dijkshoorn, 1996) before any firm epidemiological conclusions can be reached.

## Disease

Bacteria belonging to the genus *Acinetobacter* are now recognized as increasingly important opportunistic pathogens causing a wide spectrum of nosocomial infection. Although acinetobacters are generally regarded as relatively low-grade pathogens, they can cause occasional severe infection, predominantly pneumonia,

particularly in immunocompromised patients who have undergone major surgery or trauma, or those with severe underlying disease (e.g., burns, immunosuppression and malignancy). Other opportunistic infections reported include septicemia, endocarditis, meningitis, skin and wound sepsis, and urinary tract infection. Although these organisms are associated primarily with nosocomial infection, some cases of community-acquired infection have also been reported (Bergogne-Bérézin and Towner, 1996).

One of the main reasons for disease caused by *Acinetobacter* is the fact that clinical isolates of these bacteria are frequently multiply resistant to the major antibiotics used to treat nosocomial infection. Widespread resistance to the major groups of antibiotics, particularly following the recent emergence of resistance to the carbapenem antibiotics, means that *Acinetobacter* infections are difficult to treat successfully, even with combination therapy. Indeed, the extensive use of antibiotics in hospitals probably alters the normal flora and actually results in the selection of resistant strains of *Acinetobacter*. Combined with their capacity for long-term survival in the hospital environment, this makes control of outbreaks of hospital infection caused by *Acinetobacter* one of the most difficult challenges faced by infection control teams.

## Applications

A number of patent applications incorporating these organisms in various industrial processes have been awarded. An important advantage is that acinetobacters are easy to isolate, cultivate and manipulate genetically in the laboratory.

### Pollution Control

The general biochemical versatility of members of the genus has resulted in *Acinetobacter* spp. receiving considerable attention as possible industrial microorganisms. In particular, the normal soil and water habitats of many strains, combined with their ability to degrade a wide range of organic compounds, has suggested that acinetobacters can be used for bioremediation of numerous hazardous and unpleasant waste and residue pollutant compounds produced as by-products of commercial processes. Thus, aromatic compounds which are toxic to most microorganisms, such as salicylate, halogenated aromatics and phenol, are capable of being degraded by acinetobacters (Schirmer et al., 1997). In many cases, total degradation occurs by the synergic action of complex microbial communities in which acinetobacters form an impor-

tant component. Certain acinetobacters are capable of elaborating and excreting polymers (of which the most well-studied is a polyanionic, cell-associated, heteropolysaccharide termed "emulsan" produced by strain RAG-1) that emulsify hydrocarbons, such as oils (Foght et al., 1989), thus making these substrates available for degradation in an aqueous environment (Gutnick et al., 1991; Navon-Venezia et al., 1995; Barkay et al., 1999).

A more controversial area concerns the ability of acinetobacters to remove phosphate from wastewaters. Phosphate removal from wastewater to minimize the problem of eutrophication is an important feature of sewage treatment plants. It can be shown in the laboratory that certain strains of *Acinetobacter* can accumulate polyphosphates, and examination of activated sludge samples from sewage treatment plants has shown that acinetobacters form a significant part of the constituent microflora. It has therefore been postulated that acinetobacters could form the basis of an inexpensive biological method for the direct removal of phosphate from wastewater, but their precise role (and its feasibility) in such a process remains a matter of some debate.

### Biopolymers and Biosurfactants

Apart from its use in the degradation of oil, emulsan has a whole range of potential applications in the petroleum industry, including viscosity reduction during pipeline transport following formation of heavy oil:water emulsions, and production of fuel oil:water emulsions for direct combustion. The affinity of purified emulsan for the oil:water interface also has implications for the stability of oil emulsions during transport and storage, and also for their biodegradability following accidental spillage (Gutnick et al., 1991). In addition, U.S. Patent No. 4,619,825, filed on behalf of the Colgate-Palmolive Company, describes the use of emulsan-containing formulations for either mouthwash or toothpaste which have the effect of significantly reducing dental plaque formation and enhancing anti-carries activity.

A second important polysaccharide produced by a strain of *Acinetobacter* has been shown to bind to inorganic materials such as calcium carbonate (limestone). This polysaccharide has been termed "biodispersan" (Rosenberg et al., 1988) and is capable of dispersing limestone in water. Because limestone is used in a wide range of industrial processes, purified biodispersan is considered to have potential applications in manufacturing processes producing common products such as paper, paints and ceramics. A further bioemulsifying polysaccharide, termed "alasan," is produced by *Acinetobacter radiore-*

Table 2. Suggested applications for *Acinetobacter* spp. and their products.

Bioremediation of waste waters and effluents
Phosphate removal
Degradation of petrochemicals
Breakdown of organic pollutants
Production of biopolymers and biosurfactants
For prevention of dental plaque
For use in paper-making and other industries
For efficient emulsification of oil waste pollutants
For incorporation in cosmetics, detergents and shampoos
Biomass production
Single cell protein production
Manganese leaching from ores
Production of immune adjuvants
Clinical uses
Production of glutaminase-asparaginase
Production of L(-) carnitine

From Towner (1996).

*sistens* strain KA53 (Navon-Venezia et al., 1995; Navon-Venezia et al., 1998; Barkay et al., 1999).

Some other suggested uses for *Acinetobacter* spp. and their products are summarized in Table 2 and have been reviewed elsewhere (Towner, 1996).

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## The Family Azotobacteraceae

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The family Azotobacteraceae is represented by two genera, *Azotobacter* (Beijerinck, 1901a, 1901b) and *Azomonas* (Winogradsky, 1938). The removal of the genera *Beijerinckia* and *Derxia* from the Azotobacteraceae was based on rRNA cistron analysis (De Smedt et al., 1980) and rRNA cistron similarities as observed in DNA-rRNA hybridization experiments (De Vos et al., 1985). Such experiments showed that *Beijerinckia* and *Derxia* are not closely related to the genera *Azotobacter* and *Azomonas*, but that they belong to other subdivisions or groups, i.e., the alpha and the beta subclasses, respectively of the Proteobacteria (see The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition). On the other hand, investigations on rRNA similarities of various gram-negative bacteria based on  $t_{m(e)}$  values of dna-rRNA hybrids showed that *Azotobacter* and *Azomonas* are closely related to one another and to the *Pseudomonas fluorescens* rRNA branch. The latter branch or group belongs to the Superfamily I in the nomenclature of De Ley and coworkers (see The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition; De Smedt et al., 1980; and De Vos et al., 1985) or the gamma group/subdivision or subclass of the purple bacteria as defined by Woese et al. (1985a, 1985b) or the Proteobacteria, as defined by Stackebrandt et al. 1988.

Members of the Azotobacteraceae are primarily characterized as nonsymbiotic (i.e., free-living), aerobic, heterotrophic bacteria whose main property is the ability to fix molecular (atmospheric) nitrogen in a nitrogen-free or nitrogen-poor medium with an organic carbon compound (preferentially sugars, alcohols, or organic acids) as energy source. However, dinitrogen fixation is not unique to this family, as this property can be observed in quite a number of other unrelated bacteria (see The Genera *Azospirillum* and *Herbaspirillum* along with The Rhizobia in the second edition). Moreover, some representatives of this family can produce associative growth

(which is different from symbiotic growth) with higher plants. For these reasons, representatives of this family are usually called nonsymbiotic nitrogen-fixers.

### General Habitats

Representatives of the Azotobacteraceae are regular inhabitants of soil, including aerially transported dust, of water habitats, and of plant surfaces such as the external environment of roots (rhizosphere) and leaves (phyllosphere). *Azotobacter chroococcum* and *A. vinelandii* also occur in marine habitats. Some species occur in much larger numbers in the rhizosphere of higher plants than in the soil itself, and it has been shown in some cases that this associative growth is beneficial for the plant because fixed nitrogen becomes available to the plant (Döbereiner, 1966, reference is not an exact match 1968). Some investigators (see “Applications”) report the same for *Azotobacter chroococcum* and a number of agricultural crops in India.

For *Azotobacter paspali* and the grass *Paspalum notatum*, the association seems to be species-specific (Döbereiner, 1966, 1970). Also, on leaf surfaces, members of this group (especially, *Azotobacter chroococcum*) often occur as nonpathogenic epiphytic flora, particularly on older or aged leaves. The bacteria probably proliferate at the expense of the sugar-rich and nitrogen-poor exudates of the plant; the exudates act as a kind of enrichment medium (see below). For the phyllosphere, it has been suggested that the fixed nitrogen becomes available to the plant (Ruinen, 1961), but an unambiguous proof using  $^{15}\text{N}_2$  has not yet been presented.

Most species of this family occur in soil as well in water, but two *Azomonas* species, *A. agilis* and *A. insignis*, have so far only been isolated from water habitats. The pH value of the soil or water environment often governs the occurrence of a certain species. Therefore, the pH of the medium, in combination with selective carbohydrate utilization, are important factors for selective isolation of the various species in enrichment media.



Fig. 1. *Azotobacter chroococcum*. Thin section through cells demonstrating the presence of large, poly- $\beta$ -hydroxybutyrate globules as reserve material. Many cells occur in pairs in the so-called diplococcus stage. Transmission electron micrograph. Bar = 0.5  $\mu$ m.

## General Identification

Morphological and physiological properties are both very important for the identification of Azotobacteraceae. All representatives have the ability to fix nitrogen in simple media. Cells are usually large, blunt to oval in shape, 2  $\mu$ m or more in diameter. The morphology may change with various growth conditions. The cells are normally in pairs, but single cells or short chains may also occur. Motile by peritrichous or polar flagella or nonmotile. Intracellular poly- $\beta$ -hydroxybutyrate is produced (Fig. 1). Cells are Gram-negative to Gram-variable (rarely). Endospores are not formed. Microcysts are formed in one genus (*Azotobacter*). They are chemoheterotrophic, preferentially utilizing sugars, alcohols, and salts of organic acids. Cultures are obligately aerobic and catalase-positive. They fix molecular nitrogen in a nitrogen-free or nitrogen-poor medium in air, but also under reduced oxygen pressure. Organic growth factors are not required. Trace elements (e.g., molybdenum or vanadium) are required because they are involved in nitrogenase activity. Normally, cells fix 10 mg of nitrogen per g of a suitable carbohydrate (usually glucose) in synthetic media containing 1–2% carbohydrate. However, the efficiency of dinitrogen fixation can be markedly increased (sometimes doubled up to 20–25 mg N/g carbohydrate) by lowering the carbohydrate levels (Becking, 1971), to levels such as usually occur in soils, or when under low oxygen tension (Meyerhof and Burk, 1928; J. H. Becking, unpub-

lished observations; see also “Physiological and Biochemical Aspects”).

They can utilize various sources of combined nitrogen, but some species utilize nitrate poorly or not at all. Water-insoluble and water-soluble pigments or fluorescent pigments are produced by some species.

In the genus *Azotobacter*, microcysts are formed in older cultures grown with sugar as carbon source. In some species, a medium containing butan-1-ol as organic substrate (0.1–0.2 ml is added prior to pouring of the agar plates) enhances cyst formation (Tchan and New, 1984). A microcyst can be distinguished from an endospore by its characteristic structure: a central body surrounded by a cyst coat, consisting of an exocystorium and an exine. In contrast to a spore, the cell inside the cyst is similar to the vegetative form and there are no cytological changes in the cell prior to its germination (Socolofsky and Wyss, 1961; Tchan et al., 1962). The primary habitat is soil, water, and the plant rhizosphere and phyllosphere. In this family, the GC content of the DNA ranges from 52–67.5 mol%. The type genus is *Azotobacter* (Beijerinck 1901b, 567).

## Differentiation from Other Nitrogen-Fixing Bacteria

Although some rhizobia may fix nitrogen non-symbiotically, unlike *Azotobacter*, they can only do so under reduced oxygen tension. Furthermore, their cells are generally smaller than *Azotobacter* cells (*A. paspali* excepted). Moreover rhizobia need a more complex medium (supplemented with growth substances, etc.) for growth. Other nonsymbiotic nitrogen-fixing organisms have a different cell morphology and widely different physiological and nutritional requirements depending on the taxonomic group of the Prokaryote class to which they belong.

## Genus Differentiation of Azotobacteraceae

*Azotobacter*: Microcysts formed; GC content is 63–67.5 mol% ( $T_m$ ).

*Azomonas*: Microcysts not formed; GC content is 52–59 mol% ( $T_m$ ).

## Isolation

Species of the Azotobacteraceae are typical aerobic, chemoheterotrophic, dinitrogen-fixing bacteria. Therefore, any medium of suitable pH value that contains an organic carbon source, minerals (especially phosphate), some trace ele-

ments (in particular, molybdenum and/or vanadium), and no combined nitrogen is suitable for enrichment, since only organisms that can grow at the expense of atmospheric nitrogen are able to develop.

Members of this group fix dinitrogen better at low oxygen tension, probably because fixation is a reductive process (i.e., nitrogenase is inhibited or inactivated by oxygen). However, in enrichment cultures, care should be taken to provide sufficient aeration of the medium, in order to suppress the development of anaerobic or facultatively anaerobic bacteria (evident by a characteristic butyric acid smell of the enrichment culture), which may develop when the oxygen pressure is low or when the oxygen is totally exhausted. Therefore, thin-liquid layers in Erlenmeyer flasks or petri dishes are recommended to allow sufficient oxygen access. This precaution is not absolutely obligatory for enrichment of most *Azotobacter* species, since in static culture, they usually form a pellicle on the liquid surface. It is advisable to leave this pellicle undisturbed, because careless handling or shaking of the enrichment culture may rupture the pellicle and cause the cells to precipitate as a sediment, which stimulates the development of the above-mentioned anaerobic or facultative anaerobic contaminating bacteria always present in enrichment cultures.

### General Enrichment Procedures

Members of the family Azotobacteraceae can be enriched from soil or water by adding phosphate (0.1%) and an organic carbon source, usually 1–2% sugar or a calcium (or sodium) salt of an organic acid, to one of the above substrates as will be described below. Usually, an organic carbon source and phosphate are the minimum nutrients that must be added to obtain the development of Azotobacteraceae under natural conditions in soil or water. Using this principle, a number of simple enrichment procedures can be designed for the successful isolation of members of this group.

**ISOLATION OF AZOTOBACTERACEAE BY THE SOIL-PASTE PLATE METHOD** A small amount of soil (about 30–50 g) is mixed in a porcelain mortar with about 0.5–1.0 g of organic carbon (e.g., sucrose, glucose, or mannitol), about 0.5 g of chalk ( $\text{CaCO}_3$ ) to assure an alkaline reaction, 4 drops (0.12 ml) of a 10% aqueous  $\text{K}_2\text{HPO}_4$  solution, and 4 drops of a 10% aqueous  $\text{MgSO}_4$  solution. When the water content of the soil is low, some extra distilled water is also added in order to obtain a soil paste. If the soil is too sandy, or is difficult to make into paste for other reasons, sterile clay mineral (kaolinite) is added. The soil paste is transferred with a sterile knife point or

spatula to a small, low-rimmed container, watch glass, or hollow gypsum block, and the soil is firmly pressed into the hollow surface. With the aid of a knife or spatula, preferentially sterile, the surface of the soil is neatly smoothed. The small container with the soil is subsequently placed in a petri dish containing a wet piece of filter paper and incubated at 27–30°C. Care should be taken that the top of the soil does not touch the upper lid of the petri dish, thus causing anaerobic conditions. After 3–7 days incubation, glistening, slimy *Azotobacter* colonies develop on the smooth soil surface. Colonies of *A. chroococcum* turn brown in a few days.

**ISOLATION OF AZOTOBACTERACEAE BY THE SIEVED-SOIL PLATE METHOD** In this method (Winogradsky, 1932), silica-gel plates are prepared by treating a sodium-silicate solution with acid to have a complete inorganic substrate. The plates are impregnated with nitrogen-free, carbohydrate-containing nutrient solution suitable for the cultivation of Azotobacteraceae. Before impregnation, the excess salt ( $\text{NaCl}$ , when  $\text{HCl}$  is used for gel preparation) is washed out in running tap water, and then the plates are rinsed with sterile, distilled water. The solid medium is allowed to dry in an incubator. Subsequently, the plates are impregnated with medium, the superfluous medium is poured off, and the remaining water is evaporated in an incubator. The dry plates are seeded with soil crumbs; sieved soil is used preferentially (either sieved directly over the plate or distributed on the plate with a spatula) in order to obtain small soil particles and an even distribution. After incubation at 27–30°C, colonies of members of soil-inhabiting Azotobacteraceae develop on the gel around the soil particles.

The preparation of silica gel is rather laborious, but good quality agar can also be used, i.e., agar low in combined nitrogen. Other agars can also be made sufficiently low in combined nitrogen by some preparations, i.e., by fermentation of the agar in distilled water and by repeated removal and renewal of the rinsing solution. The treated agar (10–20 g/l) can be made up with Azotobacteraceae nitrogen-free medium (see below), and the nutrient agar plates inoculated with sieved soil particles in the manner mentioned above for the silica plates. As Azotobacteraceae medium, Winogradsky's nitrogen-free medium can be used. It has the following composition (g/l):  $\text{KH}_2\text{PO}_4$ , 50.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 25.0;  $\text{NaCl}$ , 25.0;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 1.0; and  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 1.0; the pH is adjusted to 7.2 with  $\text{NaOH}$ . This is a stock solution, which can be stored indefinitely at room temperature; the medium is prepared by using 5.0 ml of stock solution and 0.1 g  $\text{CaCO}_3$  per liter of distilled water (see Tchan and New, 1984). The medium

is sterilized at 120°C for 20 min. The organic substrate (10–20 g/l) is added to this organic medium. Some sugars, including glucose, must be sterilized separately before addition to the sterilized mineral medium. The medium is usually solidified with 15.0 g agar/per liter.

**ISOLATION OF AZOTOBACTERACEAE BY THE NUTRIENT SOLUTION METHOD** A complete nutrient medium is used (see e.g., Winogradsky's nitrogen-free mineral medium supplemented with a carbohydrate source as mentioned above). This medium is preferably sterilized in order to eliminate contamination from other sources. The liquid medium is usually placed in shallow layers in Erlenmeyer flasks or in petri dishes, and about 0.3–0.5 g of wet soil of the sample to be tested is used to inoculate 100–150 ml of enrichment medium.

For the investigation of distribution of members of Azotobacteraceae in water samples, usually an organic carbon source (5–20 g/l) is added to the water sample itself. Sometimes the sample is supplemented with some inorganic nutrients (particularly phosphate and molybdenum). The water sample can also be mixed with an equal volume of sterile nutrient solution of double strength. These two methods, in general, gave better results than the inoculation of a complete medium with a small amount of the water sample (e.g., 1–5 ml water sample added to 100–150 ml medium), probably because of the lower density of Azotobacteraceae in normal natural water sources compared to soil.

As already mentioned with use of nutrient solutions, the depth of the liquid layer employed should not be too high; if the liquid layers are too thick, anaerobic conditions may occur at the bottom of the flasks, resulting in growth of contaminants which inhibit the growth of Azotobacteraceae.

## Representatives of the Genus *Azotobacter*

These are soil, water, plant rhizosphere, and phyllosphere organisms; therefore, all these substrates can be used as inoculum. Many members of this genus produce copious amounts of capsular slime (polysaccharide). No endospores are formed, but some form thick-walled microcysts, which, unlike spores, are encysted vegetative cells without cytological changes prior to their germination. The cells show motility by peritrichous flagella (Fig. 2) or they are nonmotile. The cells are Gram-negative. Although some species appear to be Gram-variable (e.g., Jensen and Peterson, 1954; Kirakosyan and Melkonyan, 1964; Norris and Kingham, 1968; Johnstone,

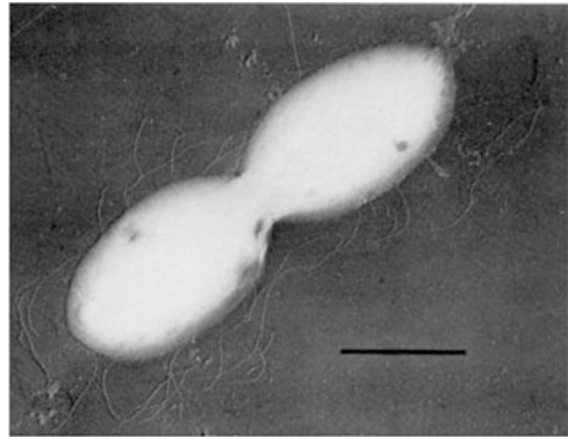


Fig. 2. *Azotobacter chroococcum*. Two cells in a pair (“diplococcus” stage) showing peritrichous flagella. Preparation shadowed with an alloy of gold and manganese. Transmission electron micrograph. Bar = 2.0  $\mu\text{m}$ .

1974), but Thompson and Skerman (1979) attribute these results to incomplete decolorization of thick smears.

## Identification of *Azotobacter* Species

Cells are motile in *Azotobacter chroococcum*, *A. vinelandii*, *A. armeniacus*, and *A. paspali*, but motility is absent in *A. beijerickii* and *A. nigricans*.

Nonmotile variants have been described in *A. vinelandii*.

Excretion of water-soluble, yellow-green, fluorescent pigment occurs in *A. vinelandii* and *A. paspali* and that of red-violet or brownish-black pigment in *A. nigricans*, *A. armeniacus*, and sometimes *A. paspali* under certain conditions.

Rhamnose as the sole source of carbon can only be utilized by *A. vinelandii* and not by any other species. For a list of other specific carbon compounds and inhibitory substances, see the specific enrichment media given below for the various species.

### *Azotobacter chroococcum*

Cells of *A. chroococcum* are pleiomorphic, bluntly rod-, oval-ovoid-, or coccus-shaped. Mean dimensions are 3.0–7.0  $\mu\text{m}$  long  $\times$  1.5–2.3  $\mu\text{m}$  wide. The cell shape changes dramatically in time or with changes in growth (medium) conditions. Cells are often in pairs. Young cells are motile by peritrichous flagella. Microcysts and capsular slime are formed. Colonies are moderately slimy, turning black or black-brown on aging. The pigment produced is not water-diffusible.

**SOIL-PASTE METHOD FOR ISOLATION OF *AZOTOBACTER CHROOCOCCUM*** The soil-paste method described under the general techniques is a quick and easy method to obtain this species from soil. Also the sieved-soil plate method and the nutrient solution method are adequate for obtaining *A. chroococcum* from soil. Apparently, because *A. chroococcum* is the most common and predominant *Azotobacter* in soil, no special enrichment techniques are necessary to make it selectively dominant.

**NUTRIENT SOLUTION FOR ISOLATION OF *AZOTOBACTER CHROOCOCCUM*** The nutrient solution method, originally described by Beijerinck (1901a, 1901b) for the isolation of *A. chroococcum*, is also a good and well-established method for enrichment and isolation of *A. chroococcum* from soil and water samples.

#### Nutrient Solution Method

To a 500-ml Erlenmeyer flask, about 100 ml of nitrogen-free nutrient solution of the following composition is added:

Distilled water	1 liter
Glucose	20.0 g
CaCO <sub>3</sub>	20.0 g
K <sub>2</sub> HPO <sub>4</sub>	1.0 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.5 g
Adjust to pH 7.2–7.6.	

In some cases it is advisable to also add trace elements, especially molybdenum (NaMoO<sub>4</sub>·2 H<sub>2</sub>O, 0.005 g/l), to the medium. The medium is inoculated with about 0.3–0.5 g of soil; for water samples, the distilled water of the prescription is replaced by the surface water tested. After 1–3 days, an *Azotobacter* pellicle forms on the liquid surface.

Due to anaerobic conditions below the pellicle, nitrogen-fixing butyric acid bacteria (e.g., *Clostridium pasteurianum*) may develop later at the bottom of the flask, and eventually throughout the medium. It is therefore advisable to subculture the *Azotobacter* pellicle according to the normal procedures (by applying appropriate dilutions in sterile tap water) as soon as possible to nitrogen-free agar plates in order to reduce contamination. Such subculturing is also advisable because combined nitrogen produced by *Azotobacter* development may make it possible for non-dinitrogen-fixing contaminants to develop.

The following agar medium can be used for the isolation, purification, and further subcultivation of *Azotobacter chroococcum* found in the enrichment cultures:

#### Agar Medium for *Azotobacter chroococcum*

Distilled water	1 liter
Glucose	20.0 g
K <sub>2</sub> HPO <sub>4</sub>	0.8 g
KH <sub>2</sub> PO <sub>4</sub>	0.2 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.5 g
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.10 g (or 0.05 g)
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.05 g

or CaCO <sub>3</sub>	20.0 g
NaMoO <sub>4</sub> ·2H <sub>2</sub> O	0.05 g
Agar	20.0 g
Adjust to pH 7.4–7.6.	

With CaCl<sub>2</sub>, the agar medium is translucent; with CaCO<sub>3</sub> it is opaque white. The latter substance is, however, in some cases an advantage for detecting *Azotobacter* colonies, because they do not produce acid on it. Acid-producing colonies, as evident by the dissolution of the calcium carbonate around the colony, are certainly not *Azotobacter* species. A further advantage for adding CaCO<sub>3</sub> to the medium is that it has a good buffering capacity at the alkaline side; an alkaline reaction is necessary for the development of *Azotobacter chroococcum*.

The presence of *A. chroococcum* in soil or water is strongly governed by the pH value of these substrates. In an environment below pH 6.0, *Azotobacter* is generally rare or totally absent (see Becking, 1961). H. L. Jensen (1965) tested 264 Danish soils and found that practically all of the soils above pH 7.5 contained *Azotobacter* (predominantly *A. chroococcum*) varying in numbers between 10<sup>2</sup> and 10<sup>4</sup> per gram of soil. Of the 148 tropical soils tested by Becking (1961), all soils above pH 7.5 (pH range 7.5–9.0) contained *Azotobacter* (also mainly *A. chroococcum*) and, in the pH ranges of 7.0–7.4, 6.5–6.9, and 6.0–6.4, the percentage of *Azotobacter*-positive soils was 89, 57, and 32%, respectively. In nitrogen-free nutrient media, the lower pH limit for growth of *A. chroococcum* strains in pure culture is between pH 5.5 and 6.0 (Jensen and Petersen, 1955).

As already mentioned earlier (Becking, 1962), according to the author's experience, not all *Azotobacter* strains that produce a brown or brown-black pigment on aging (Fig. 3) belong to one species, because *Azotobacter chroococcum* strains are very pleomorphic (see Fig. 4). It was therefore likely that this species comprises a more complex group consisting of several species, whose delimitations have not yet been sorted out. The latter is confirmed by Thompson and Skerman (1979) who examined 151 strains of Azotobacteraceae of different provenance in 230 variant tests and analyzed the data according to numerical methods in a hierarchical classification of groups. They obtained evidence that at least two distinct black-brown-pigmented *Azotobacter* species occur next to *A. chroococcum*, i.e., *A. nigricans* and *A. armeniacus* (see below).

#### *Azotobacter nigricans*

This species, originally isolated by Krasil'nikov (1949), possesses, in contrast to *A. chroococcum* (but like strains of *A. beijerinckii*), nonmotile

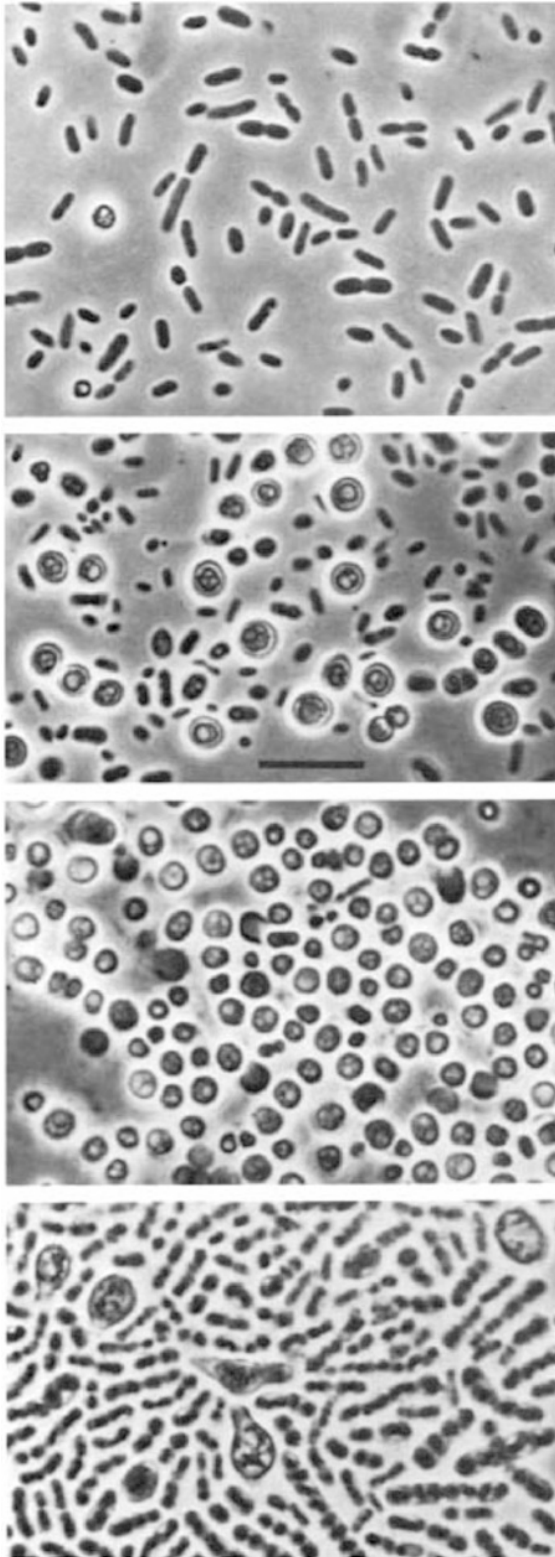


Fig. 3. *Azotobacter chroococcum* cells. Four different strains of the same age growing on an identical medium (nitrogen-free, mineral glucose agar with 2% calcium carbonate). Note the presence of lipoid-filled cells, cysts, and germinating cysts in some of the strains. Living preparations; phase contrast micrographs. Bar = 10  $\mu\text{m}$ .

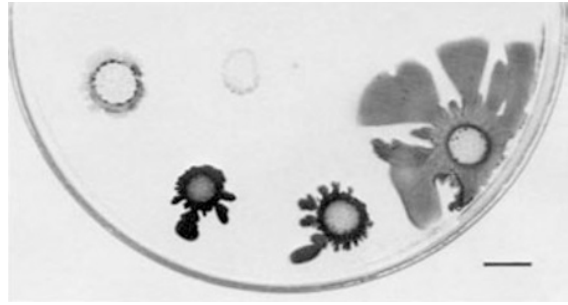


Fig. 4. *Azotobacter chroococcum* colonies. Strains growing on the same medium (nitrogen-free, mineral glucose agar with calcium carbonate) and of the same age, showing the variation of the chromogenesis of the colonies. Bar = 1 cm.

cells. Cells are bluntly rounded rods in shape, occurring singly or in pairs. Mean cell dimensions are 4.1–4.9  $\mu\text{m}$  long  $\times$  1.5–2.7  $\mu\text{m}$  wide. Moreover, they differ from *A. chroococcum* in a number of nutritional properties, such as the inability to utilize ethanol, pentan-1-ol, propionate, caproate, and benzoate and to produce brown-black or red-violet diffusible pigments. They differ from *Azotobacter armeniacus* by the inability to utilize caprylate, which compound can be used as carbon source by the latter species. In *A. nigricans*, the production of agar-diffusible homopolysaccharides from sucrose or raffinose is strain variable, but no strain produces any colony-retained homopolysaccharide. On aging, colonies of *A. nigricans* turn black-brown due to the same diffusible pigment, but a variant formerly called *A. beierinckii* subsp. *achromogenes* Jensen and Petersen 1954, but now proposed to be *A. nigricans* subsp. *achromogenes* (Thompson and Skerman, 1979) produces only a yellow non-diffusible pigment within the colony and no diffusible pigment.

Strains of this species has been isolated from European soils, particularly from East European soils.

There is no species-specific selective enrichment method for isolating *A. nigricans*.

#### *Azotobacter armeniacus*

This is a poorly understood species that has been described by Thompson and Skerman (1981). Cells are bluntly rounded rods, occurring singly or in pairs. Mean cell dimensions are 5.0–5.7  $\mu\text{m}$   $\times$  1.7–2.0  $\mu\text{m}$ . Strains of this species differ from *A. nigricans* by having motile cells, but they have in common with the latter species the production of a diffusible brown-black or red-violet pigment into the medium. In contrast to *A. nigricans*, they are able to use citrate or D-galacturonate as sole carbon source and most strains also can use *n*-valerate and caprylate, but like *A. nigricans*,

capronate is not utilized. All *Azotobacter* species, including *A. nigricans*, can utilize ammonium and nitrate as sole source of nitrogen, and these N-sources are assimilated in preference to molecular nitrogen, *A. armeniacus* is unable to use ammonium, nitrate, or glutamate as sole source of nitrogen for growth (Thompson and Skerman, 1979).

There is no special enrichment medium for *A. armeniacus*, but probably it can be enriched with caprylate as sole source of carbon, but *A. vinelandii* also can be grown on this medium. Further isolation, however, will differentiate between these two species.

### *Azotobacter beijerinckii*

Formerly, this organism was not regarded as a distinct species, but merely as a nonpigmented variant of *A. chroococcum*. In the eighth edition of *Bergey's Manual* (Buchanan and Gibbons, 1974), it has, however, been restored to the species level. Cells are bluntly ended rods or ellipsoidal, occurring singly or in pairs, sometimes in short chains. Mean cell dimensions are 3.2–5.3  $\mu\text{m}$  long  $\times$  1.7–2.7  $\mu\text{m}$  wide.

The main differences between *Azotobacter beijerinckii* and *A. chroococcum* are that colonies of *A. beijerinckii* produce, on aging, a yellowish or cinnamon pigment (in *A. chroococcum*, it is brown or blackish-brown) and its cells are nonmotile (motile in *A. chroococcum*). Moreover, it invariably lacks the ability (in contrast to *A. chroococcum*) to utilize starch as the sole source of carbon. Sometimes the inability to utilize mannitol is also mentioned as a determinative character (see Johnstone, 1974), but 9 out of 10 strains tested by Jensen and Petersen (1955) showed good growth on this carbon source. Thompson and Skerman (1979) mentioned in addition, that caproate is utilized by *A. chroococcum*, but not by its satellite species, *A. nigricans* and *A. armeniacus*. The differences between *A. nigricans*, which is also a species with nonmotile cells, and *A. beijerinckii* are not so clear with respect to carbon source utilization, except that malonate is used by all strains of *A. beijerinckii*, but only by a minority of strains of *A. nigricans*, and that D-glucuronate, D-galacturonate, and benzoate are utilized by *A. beijerinckii* but not by *A. nigricans* (Thompson and Skerman, 1979). The main difference remaining is the pigment, which in *A. nigricans* is black-brown to red-violet, whereas the excretion of any water-soluble pigment into the substrate is completely absent in *A. beijerinckii*. The physiological differences between *A. chroococcum* and *A. nigricans* or *A. armeniacus* are already outlined under the headings of the latter two species.

Tchan (1953) described a variant of *A. beijerinckii*, named *A. beijerinckii* subsp. *acidotolerans*, which could grow and fix dinitrogen at a pH of 4.75. Later, Tchan and New (1984) delineated this characteristic more in detail and distinguished two subgroups. An acid-tolerant subgroup and another subgroup distinguished by its sensitivity to 0.05% phenol or 40  $\mu\text{g}/\text{ml}$  diamond fuchsin, its inability to utilize sorbitol or aconitate, and its failure to produce diffusible homopolysaccharides. V. Jensen and Petersen (1954) have also described another form of *A. beijerinckii*, *A. beijerinckii* subsp. *achromogenes*, in which under the cultural conditions applied, no pigment ever was produced. The latter strains were isolated from Danish calcareous forest soils in a survey including all types of Danish soils (see later).

Jensen and Petersen (1954) also showed that acid tolerance is a rather common characteristic of *A. beijerinckii*, since all strains isolated could grow and fix dinitrogen to a pH of 5.1. The nitrogen-fixation data published by Jensen and Petersen (1955) showed, however, that considerably more atmospheric nitrogen was fixed under alkaline than under acidic conditions.

For selective enrichment of *A. beijerinckii* and elimination of *A. chroococcum*, a selective nitrogen-free medium of the same composition as used for *A. chroococcum*, but with  $\text{CaCl}_2$  (instead of  $\text{CaCO}_3$ ) and with a slightly acid pH (pH 4.9–5.5), can be employed. So far, no experience has been gained with such a medium since all *A. beijerinckii* strains so far known are casual isolates coming from *A. chroococcum* plates. It might be surmised that *A. beijerinckii* would favor somewhat acidic soils. In this respect it is remarkable that, in the above-mentioned survey of Danish forest soils by V. Jensen and Petersen (1954), *A. beijerinckii* was found predominantly in soil samples of two localities with beech (*Fagus sylvatica*) forest on calcareous soil with pH values of 7.0–8.0 and 7.8–8.0, respectively. In these and nearly all other forest soils tested, *A. chroococcum* was absent or nearly absent. These observations indicate that some calcareous forest soils are a favorable and probably selective habitat for *A. beijerinckii* and that the occurrence of *A. chroococcum* is confined to the more alkaline agricultural soils.

### *Azotobacter vinelandii*

Cells are rounded-ended rods, occurring singly or in pairs. Mean cell dimensions are 3.0–4.5  $\mu\text{m}$  long  $\times$  1.5–2.4  $\mu\text{m}$  wide (Fig. 5). Cells are motile with numerous peritrichous flagella; very rarely, some are nonmotile. Colonies are nonpigmented. They excrete a yellow-green, fluorescent, water-soluble pigment into medium.

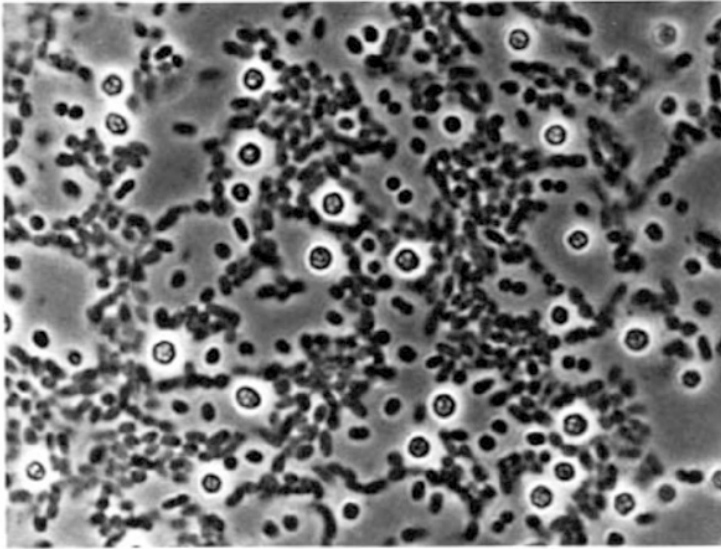
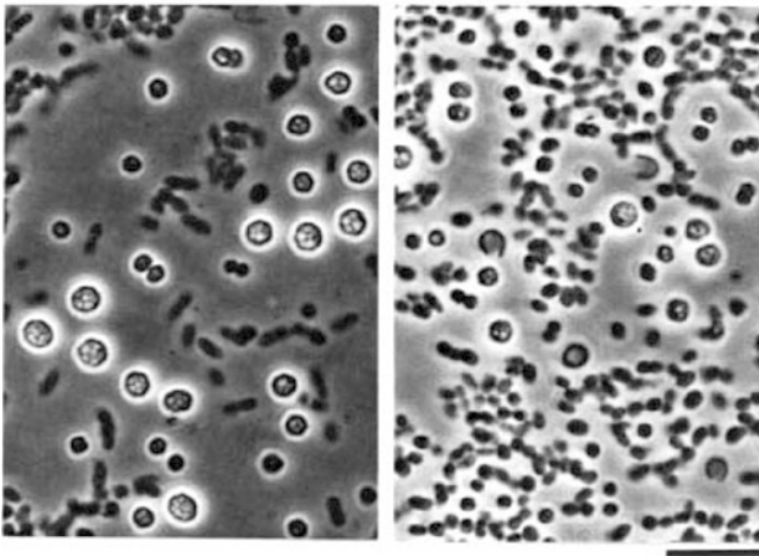


Fig. 5. *Azotobacter vinelandii* cells. Three different strains growing on the same medium (nitrogen-free, mineral glucose agar) showing some variation in the cell size of the strains and in the number of lipid-filled cells, cysts, and germinating cysts. Living preparation, phase contrast micrographs. Bar = 10  $\mu$ m.



Fewer strains of *Azotobacter vinelandii* have been isolated in comparison with strains of *A. chroococcum* and *A. beijerinckii*. Most of the strains were casual isolates obtained from soil, such as the original strain of Lipman (1903a) from New Jersey (USA) soil (pH unknown). Wilson's strain O(P), used for many physiological and biochemical studies (e.g., Wilson and Knight, 1947; Shutter and Wilson, 1955), is probably a subculture of this strain. Bortels (1930) isolated another strain from German soil (originally described as *Azotobacter agile*), and Winogradsky (1932) obtained one strain from soil in France. Later, Winogradsky (1938) also underlined the relative rarity of this species.

The present author has obtained a number of strains—often in association with *A. chroococcum*—from Dutch soil, but also from freshwater samples using the nutrient solution method for

*A. chroococcum* (preferentially with ethanol as carbon source) for enrichment (J. H. Becking, unpublished observations). *A. vinelandii* was obtained mostly from rather alkaline Dutch and European soils, such as calcareous soils and soil derived from marine sediments, sea sludge, or sea muds that had been pumped up for leveling land. The latter soils, usually also rather rich in sodium chloride, showed pH values of 8.0–9.5.

The water samples from which this organism was obtained were pond, lake, or marsh water of alkaline reaction (pH 7.5–8.0). This species was also numerous in some tropical soils, such as alkaline sea muds and mainly calcareous soils of Indonesia and certain very alkaline soils of Bolivia and other localities in South America (Becking, 1961; J. H. Becking, unpublished observations). Clearly, alkaline conditions are favorable for the occurrence of *A. vinelandii* and



probably selective for its distribution. Further, this species may be halophilic to some degree, or at least resistant to salinity, in view of isolation from alkaline sea muds. The presumed halophilic properties need, however, experimental confirmation.

Derx (1951b) designed a method for selective isolation of this species from soil and water sources. The underlying principles of this method were the addition to the enrichment medium of sodium benzoate (1.0%) as an inhibitor in order to suppress the development of *A. chroococcum*, and the use of a special carbon source, such as mannitol or ethanol, which is very readily assimilated by *A. vinelandii*. Derx's method was a further extension of earlier work of Reuszer (1939), who observed that by applying benzoate, benzoic acid, or another phenolic compound to soil, the normal population of *Azotobacter chroococcum* and *A. beijerinckii* was replaced completely by a green-pigment-producing form of *Azotobacter*, which had not been found before in the soil.

#### Derx's Medium for Selective Enrichment of *Azotobacter vinelandii* (Derx, 1951b)

Distilled water	100 ml
Mannitol	0.5 g
or ethanol	1.0 ml
K <sub>2</sub> HPO <sub>4</sub>	0.5 g
Sodium benzoate	1.0 g
Adjust to pH 7.5–8.0.	

For the isolation of this species from soil, ethanol is preferentially used as carbon source; for its isolation from water samples, the use of mannitol as carbon source is recommended since *Azomonas agilis* (see "Representatives of the Genus *Azomonas*") is usually unable to assimilate this compound. According to the present author, the rather alkaline reaction induced by the addition of sodium benzoate may also be responsible for the selective properties of this medium for the enrichment of *A. vinelandii*.

Numerous strains of *A. vinelandii* were obtained by the present author from Dutch water habitats, such as the Rhine River, and pond and lake water (usually of pH 7.0–7.5) by using Derx's medium with mannitol as carbon source and the supplement of sodium benzoate. In all cases, the enrichment medium was adjusted to pH 8.0–9.0. Use was made of 300-ml Erlenmeyer flasks with 50 ml of medium or 100-ml Erlenmeyer flasks with 20 ml of medium. For the water samples, the sample itself, instead of distilled water, was used as liquid to which the carbon source and salts were added.

V. Jensen (1961) mentioned that L-rhamnose (1.0%) could be used as a carbon source for the selective enrichment of *A. vinelandii*, since only a very small fraction of the strains of the other *Azotobacter* and *Azomonas* species tested could

utilize this compound. When grown in pure culture on L-rhamnose, *A. vinelandii* develops profusely within 3–5 days at 25°C, while the other *Azotobacter* species that can utilize this compound only produce some development after 1–2 weeks. Therefore, in spite of the presence of other *Azotobacter* species, *A. vinelandii* soon becomes dominant in such an enrichment medium.

Using the L-rhamnose enrichment medium, V. Jensen (1961) observed *A. vinelandii* to be sparsely distributed in normal soil. It was only present in a very few garden soils and seemed to be restricted to the most fertile soils. Although Johnstone (1974) reported that *A. chroococcum* and *A. beijerinckii* do not utilize L-rhamnose as sole source of carbon, according to the present author, this is not always true. Utilization of L-rhamnose is not a common feature of strains of these species, when these are obtained as random isolates with the more common sugars, but some strains utilizing this carbon source can be secured in enrichment media by using L-rhamnose as sole source of carbon. Also, Thompson and Skerman (1979) observed that one of the 19 *A. chroococcum* strains tested could utilize L-rhamnose, while Claus and Hempel (1970) isolated both *A. chroococcum* and *A. beijerinckii* from soil and water samples with L-rhamnose as sole substrate.

Claus and Hempel (1970) observed that resorcin, ethylene glycol, or glutarate, all in 0.1 or 0.2% (wt/vol) concentration, are very selective carbon sources for *A. vinelandii*, and that a number of strains could be isolated from soil by using these compounds as sole source of carbon in enrichment media. The above-mentioned carbon compounds apparently cannot be utilized by the more common *Azotobacter* species.

*A. vinelandii* strains have numerous peritrichous flagella, in general, but Derx (1951b) isolated nonflagellated strains of *A. vinelandii* for which he proposed the name *Azotobacter non-vinelandii*, which name is, however, considered not to be validly published under Rule 23 of the Bacteriological Code (reference is not an exact match year Lapage et al., 1975). Another characteristic of these strains, which distinguishes them from typically flagellated strains of *A. vinelandii*, is the ability to produce a black pigment in the presence of benzoate (Derx, 1951b).

In multiple tests for numerical analysis, Thompson and Skerman (1979) confirmed that all *A. vinelandii* strains tested could readily utilize rhamnose as sole source of carbon, in contrast to most strains of other species. Moreover, they observed that *A. vinelandii* could utilize caproate, caprylate, and meso-inositol, compounds which generally are not utilized by the other *Azotobacter* species. Furthermore, 0.1%

phenol can be used in enrichment cultures to inhibit the growth of other *Azotobacter* species, and incubation at 37°C particularly favors the development of this species. According to these authors, the nonmotile *Azotobacter non-vinelandii* strains may be regarded as a special subgroup that differs from typical *A. vinelandii* strains by the inability to utilize raffinose and by the utilization of pimelate, suberate, and sebacate as sole sources of carbon. In addition, in contrast to typical *A. vinelandii*, these strains were resistant to brilliant green at a concentration of 10 µg/ml.

The presence of *A. vinelandii* in enrichment media can be detected by a color change of the medium, which turns yellow, green, or violet. On nitrogen-free, mineral agar plates, a water-soluble yellow, green, or violet fluorescent pigment is excreted into the medium, but they do not produce any nondiffusible pigment. Pigment production into the substrate is stimulated by low-iron concentration or iron deficiency of the medium (Becking, 1962). As shown by Johnstone (1955, 1957b) and Johnstone and Fishbein (1956), the fluorescence of the pigment under ultraviolet light shifts with pH changes, and different fluorescence curves are obtained with the diffusible pigments of *A. vinelandii* and *Azomonas agilis*. Thus, fluorescence measurements also have diagnostic value in distinguishing between both species.

### *Azotobacter paspali*

Cells are long, bluntly ended rods. Mean cell dimensions are 7.0–10.9 µm long × 1.3–1.7 µm wide; sometimes shorter rods of 3.2–4.2 µm long × 1.6–1.9 µm wide are seen. Cells in young cultures often form long filaments. A yellow-green, fluorescent or red-violet, water-soluble pigment is excreted into the medium.

This species, described by Döbereiner (1966), was originally isolated on Winogradsky's silica-gel plates impregnated with a mineral salt solution (see sieved-soil plate method) with a pH of 6.5 and calcium citrate as sole source of carbon. Later (Döbereiner, 1970), a N-free, mineral sucrose agar (a modification of Lipman's [1903b, 1905] medium) was recommended, because it gave satisfactory results and it was much easier to prepare.

#### Nitrogen-free, Mineral Sucrose Agar for Isolation of *Azotobacter paspali* (Döbereiner, 1970)

Distilled water	1 liter
K <sub>2</sub> HPO <sub>4</sub>	0.05 g
KH <sub>2</sub> PO <sub>4</sub>	0.15 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.20 g
CaCl <sub>2</sub>	0.02 g
CaCO <sub>3</sub>	1.0 g
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.002 g

FeCl <sub>3</sub> (10%, aqueous solution)	1 drop
Bromthymol blue (0.5%, ethanol solution)	10 ml
Sucrose	20 g
Agar	20 g

Adjust the pH to ca. 7.0.

Plates with this medium were inoculated with root-surface (so-called rhizoplan) soil of a grass species, *Paspalum notatum*. The soil (about 20–50 mg per plate) was usually scattered directly from the roots on the plates. However, the soil also can first be passed through a sieve (0.5-mm mesh), and then 50 mg can be weighed out and scattered over the plates for quantitative tests. The plates were incubated at 35°C.

Differentiation of *A. paspali* from the other *Azotobacter* species on silica-gel plates with calcium citrate as carbon source is relatively easy. Colonies appear 4–5 days after inoculation and these produce an intense yellow pigment, which diffuses into the silica gel below the colony. The colonies are relatively small and raised and readily solubilize the opaque white calcium citrate layer at the top of the silica gel, giving the appearance of many little holes. Later, the colonies spread rapidly and become flat.

On the above-mentioned agar medium, *A. paspali* colonies appear 2–3 days after inoculation and incubation at 37°C. The colonies are dense, raised, and yellow in color due to acid production, because bromthymol blue is added as an indicator to the medium.

The acid-producing ability of this species is not found in any other *Azotobacter* species. Moreover, the ability of *A. paspali* to use organic compounds (only 17 out of 159 tested by Thompson and Skerman, 1979) is much more limited than that of other *Azotobacter* species. *A. paspali* cells are usually 7–12 µm long and 1.3–1.7 µm wide, but occasionally exceptionally long rods (up to 60 µm × about 2.0 µm) are seen. These are typical for this species and are a significant morphological feature, which is not seen in any other *Azotobacter* species. A further characteristic of this species is that cells of the microcolonies are often dimorphic, some colonies possess cells 7–11 µm long, whereas other colonies have cells 3–4 µm in length (Thompson and Skerman, 1979). Cells of older colonies on nitrogen-free agar may reach a length of 60 µm (see above).

From numerical analysis, Thompson and Skerman (1979), concluded that *A. paspali* is not closely related to other *Azotobacter* species. Moreover, they observed an antagonism toward Gram-positive bacteria in this species. In addition, their very restricted habitat—they occur solely in the plant rhizosphere—is unique among all the other *Azotobacter* species. In view of these differences, these authors proposed a new genus for this species, *Azorhizophilus* (Thompson and Skerman 1981). This opinion is, however, opposed by (De Smedt et al. 1980), who found

that the rRNA cistron of *A. paspali* is almost identical with those of *A. chroococcum*, *A. beijerinckii*, *A. vinelandii*, and *A. nigricans*. Moreover, Tchan et al. 1983, using rocket-line immunoelectrophoresis, showed that *A. paspali* is not immunologically separable from other members of this genus. In view of this, it is not desirable to place *A. paspali* in a separate genus.

Strains of *A. paspali* excrete a diffusible, yellow green, in UV-light fluorescent pigment into the substrate, particularly on iron-deficient media.

The association between *Azotobacter paspali* and the grass, *Paspalum notatum*, is highly species-specific; this association is found only in a few other *Paspalum* species (i.e., *P. plicatulum*, *P. dilatatum*, and *P. virgatum*) of 16 other *Paspalum* species tested for this bacterium (Döbereiner, 1970). It was never found in other Gramineae examined and in some Leguminosae and other dicots tested.

The original isolate came from a broad-leaved and hairy *Paspalum notatum* variety "Batatais" (common Bahia grass) in Brazil, but it was later also found in the same and other *Paspalum* species and varieties in Florida (USA) and Puerto Rico, but not in rhizosphere soil of *Paspalum* from Argentina, Paraguay, and South Africa (Döbereiner, 1970). However, according to the present author, no definite conclusion from its absence in the latter three regions can be made, because only a very restricted number of rhizosphere soil samples (one to five) of these localities have actually been examined.

## Representatives of the Genus *Azomonas*

Members of the genus *Azomonas* are primarily aquatic. *Azomonas agilis* and *A. insignis* are obligately aquatic organisms, since so far they have only been isolated from freshwater habitats. The only exception is *A. macrocytogenes*, which has been isolated from soil. The latter species was first isolated and described by H. L. Jensen (1955). For a long time, it was only known from a single strain and two variants derived from it, but according to Thompson and Skerman (1979) a total of 7 strains is now available. However, probably all, except one strain, are derivatives or subcultures of Jensen's original strain!

*Azomonas* species differ from those of the genus *Azotobacter* by a number of morphological and physiological characteristics. They all possess relatively large cells, which frequently occur singly and which have a special type of flagellation that gives the cells a high motility. Microcysts are never formed. Colonies on agar are generally opaque, glistening, smooth, and without insoluble pigment. On iron-deficient

agar media, a yellow-green diffusible pigment is formed; and in some other media, often more red-violet or purple diffusible pigments are produced. Usually pigment production is very pronounced in liquid media and less pronounced on solid media. In *A. agilis* and *A. macrocytogenes*, the pigments are fluorescent in UV light; *A. insignis* has a nonfluorescent pigment.

## Identification of *Azomonas* Species

Apart from cell shape and dimensions, which will be covered later in the descriptions of the separate species, *A. macrocytogenes* can readily be distinguished by the formation of enlarged filamentous cells in media with ethanol, a phenomenon absent in *A. agilis* and *A. insignis*. Moreover, flagellation is peritrichous in *A. agilis*, lophotrichous in *A. insignis*, and usually monotrichous (sometimes, however, two flagella at one pole) in *A. macrocytogenes*. With regard to carbon sources as sole source of carbon. It has been observed that mannitol is utilized by *A. macrocytogenes* and not by *A. agilis* and *A. insignis*, and malonate gives just the reverse outcome, i.e., it is utilized by *A. agilis* and *A. insignis*, but not by *A. macrocytogenes*. Finally, maltose is readily utilized by *A. macrocytogenes*, but not by *A. insignis* and only by a minority of the *A. agilis* strains. All species produce water-diffusible pigments, but in contrast to the pigments of *A. agilis* and *A. macrocytogenes*, the pigment of *A. insignis* does not produce fluorescence in UV light.

### *Azomonas agilis*

This species has large, ovoid, ellipsoidal or coccoid cells, often giving it a protist-like appearance (Fig. 6). Cells are seldom found in pairs. Cells are usually 2.5–6.4  $\mu\text{m}$  long and 2.0–2.8  $\mu\text{m}$  wide; sometimes giant cells up to 10.0–13.5  $\mu\text{m}$  long have been observed (Fig. 6). The cells are motile by means of peritrichous flagella. No microcysts are formed. A nondiffusible pigment is not produced, but a diffusible yellow-green or red-violet pigment, particularly on iron-deficient media. In UV light, the pigment gives a bluish-white fluorescence.

This species was first isolated and described by Beijerinck (1901a, 1901b), who obtained it from Dutch (Delft) canal water. Beijerinck used the following enrichment medium:

Enrichment Medium for *Azomonas agilis*  
(Beijerinck, 1901a, 1901b)

Canal water	100 ml
Mannitol	2.0 g
K <sub>2</sub> HPO <sub>4</sub>	0.02 g

The incubation temperature is 25–30°C.

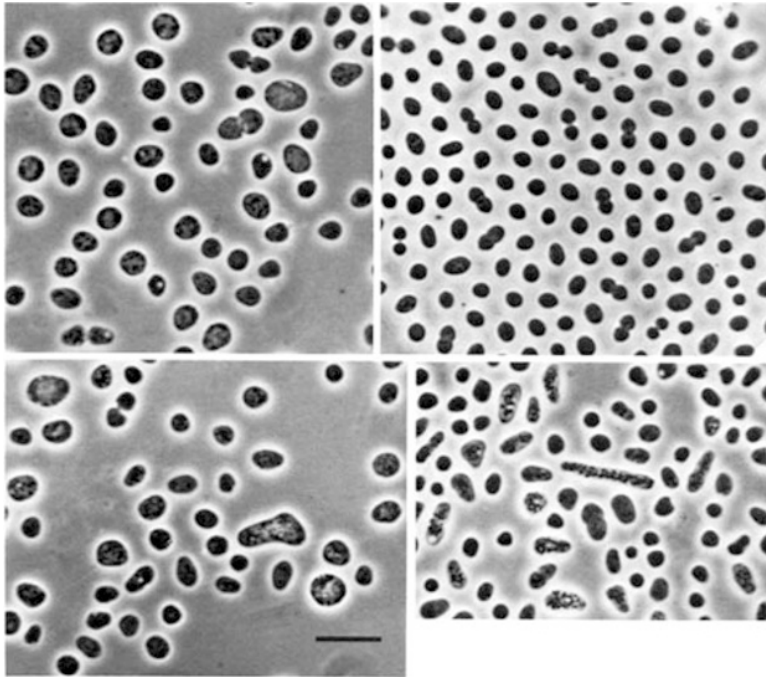


Fig. 6. *Azomonas agilis*. Cells of two strains demonstrating the large, protist-like appearance of the cells and the sporadic occurrence of aberrantly shaped, pleomorphic cells. Living preparations, phase contrast micrographs. Bar = 10  $\mu$ m.

Later, Kluver and van Reenen (1933) and Kluver and van den Bout (1936) obtained other strains with the same method. A strain isolated by the latter authors from Delft canal water (ATCC 7494) is now the neotype, because Beijerinck's original strain has been lost. It is remarkable that *A. agilis* in pure culture cannot utilize mannitol, so this carbon source must first be degraded by other microbes before it can become available to *A. agilis*. It is also possible that immediately after isolation, the strains can actually utilize mannitol, but that this ability is later lost during purification and therefore absent in pure cultures. Kluver and van Reenen (1933) observed that, when cultures were streaked immediately from the enrichment medium on plates containing tap water, 2% mannitol, 0.02%  $K_2HPO_4$ , and 1.5% agar, and incubated at 20°C, *A. agilis* develops in 2–3 days to very small colonies about 1 mm in diameter. Growth on agar plates that contain glucose (2%) instead of mannitol as source of carbon was luxurious and with much slime production, but isolation from these plates was not recommended because many accompanying contaminants may develop in the capsular slime produced.

Derx (1951b) designed specific methods for the enrichment of *Azomonas agilis* by eliminating, so far as possible, the development of the more common *Azotobacter* species and of other *Azomonas* species. The underlying principle of Derx's method is that *Azomonas agilis*, in contrast to the more common *Azotobacter* species, is remarkably tolerant to the presence of 1%

sodium benzoate in the medium, although it cannot utilize this compound as a carbon source. Derx also added a carbon compound readily utilizable by *A. agilis*. Mannitol cannot be used as the carbon source because it is normally not utilized by *A. agilis*, but it is assimilated by *Azotobacter vinelandii*. The latter organism is frequently also present in water samples and, moreover, is resistant to 1% sodium benzoate. Therefore, Derx recommended the use of ethanol as sole source of carbon for the isolation of *Azomonas agilis*. The following enrichment medium gave good results.

#### Selective Medium for *Azomonas agilis* (Derx, 1951b)

Water sample (canal, river, or lake water)	100 ml
Ethanol	1 ml
$K_2HPO_4$	0.05 g
Sodium benzoate	1.0 g

The enrichment medium is placed in relatively thin layers in Erlenmeyer flasks to allow good oxygen access, e.g., 10–15 ml of medium in 100-ml flasks, or 50 ml medium in 250-ml flasks.

Following Derx's method, the author was able to isolate a large number of *Azomonas agilis* strains from various water sources. The organism proved to be particularly common in strawboard factory wastewater, as already observed by K. T. Wieringa (personal communication; see also Smit, 1954) and by Johnstone (1957a), and in some heavily polluted waters. In clear and clean water of rivers, lakes, and turbulent or rapidly moving, oxygen-rich, mountain brooks and rivu-

lets, it was rarely present and frequently totally absent. In this habitat, it is entirely replaced by *Azomonas insignis*.

The tolerance of *A. agilis* strains to salt concentration up to 1.0% suggest that they are able to live in contaminated waters where concentrations of organic matter and mineral salts can be relatively high. Also its resistance to iodoacetate (1  $\mu$ M) points in this direction, and this compound could be used for the selective enrichment and isolation of this species (Thompson and Skerman, 1979).

In agreement with this general pattern of being able to survive in polluted environment, nitrate is never or rarely reduced to nitrite by strains of *A. agilis* in contrast to strains of *A. insignis*. Growth experiments using *A. agilis* strains on nitrate-containing media incubated in air revealed that nitrate is an inert, nonutilizable compound for them, and thus they are able to fix molecular nitrogen in the presence of nitrate in the medium (Becking, 1962). In 19 out of 20 *A. agilis* strains tested, vanadium was unable to replace molybdenum in nitrogen fixation (Becking, 1962), indicating that they predominantly have only a molybdenum-activated nitrogenase system.

### *Azomonas insignis*

Colonies on nitrogen-free media are usually small, smooth, translucent, and low convex. They show a low proportion of extracellular polysaccharides. Cells are round or ellipsoidal and are usually 2.5–3.9  $\mu$ m in length and 1.7–2.6  $\mu$ m in width. On average, they measure 3.1  $\times$  1.9  $\mu$ m (Derx, 1951a). The cells have lophotrichous flagella. No microcysts are formed. On iron-deficient media, a yellow-green, red-purple, or violet pigment may be produced. The pigments are, however, not UV fluorescent.

As will be shown in the enrichment methods for this species, ethanol and salts of organic acids are preferential carbon sources for the isolation of *A. insignis*. On the whole, the utilization of organic carbon compounds as sole source of carbon for growth and energy is very restricted in this species (see later). The type strain of Derx (1951a), which is no longer extant, could not utilize glucose as sole source of carbon. It was probably lost by subcultivation on glucose-containing agar slants. New isolates (see V. Jensen, 1955) could utilize glucose, but with regard to carbohydrate utilization, in all strains tested, it is limited to only two, D-glucose and D-fructose (Thompson and Skerman, 1979).

This species was first isolated and described by Derx (1951a). It was obtained from water of clear, rapidly flowing mountain streams and small rivulets of the Cibodas Nature Reserve,

Western Java, Indonesia (ca. 1,400 m altitude). These rather turbulent streams have limpid, oxygen-rich water, which contains much soluble calcium. *Azomonas agilis* could never be isolated from these sources (see above).

Derx (1951a) recommended the following medium for the specific enrichment of *A. insignis* from these water sources:

#### Selective Medium for *Azomonas insignis* (Derx, 1951a)

Water sample	100 ml
Calcium formate	0.5 g
Ethanol	1.00 ml

The enrichment medium is placed in rather thin layers in Erlenmeyer flasks, i.e., 20–25 ml in 100-ml flasks or 50 ml in 250-ml flasks. Incubation is at 25–30°C. Calcium formate is added to the medium to inhibit the development of various other microbes, including *Azotobacter* species and other *Azomonas* species. *A. insignis* does not utilize calcium formate but is rather resistant to it. Ethanol is added as sole source of carbon because it is a preferential carbon source for *A. insignis*. According to Derx (1951a), his strains of *A. insignis* could not utilize glucose or mannitol. A nonvolatile carbon source, which also gives good growth and can be used for maintenance in pure culture on agar slants is calcium malate or calcium succinate. Therefore, the use of nitrogen-free, mineral agar plates with calcium malate (0.5%) or calcium succinate (0.5%) as sole carbon source can also be recommended for isolation of *A. insignis* from enrichment cultures.

Pure cultures can also be grown in liquid medium: tap water (for trace element supply), to which is added  $K_2HPO_4$  (0.05%) and ethanol (0.5–1.0%, or about 0.5–1.0 ml per 100 ml medium). Growth is obtained in 24 h at 25–30°C. After several days, the medium turns milky white, later blue-gray, and after about a week sometimes to violet (see above).

V. Jensen (1955) isolated six strains of *A. insignis* from surface water of fast-flowing brooklets and streams with very limpid fresh water in Denmark, but occasionally also from stagnant and somewhat polluted water (e.g., his strains 7 and 9). None of his isolates could utilize mannitol but, in contrast to Derx's strains, all the Danish strains could utilize glucose as sole source of carbon. Two of Jensen's strains were also able to grow in a medium that contained sodium benzoate (1.0%).

#### Enrichment Medium for *Azomonas insignis* (V. Jensen, 1955)

Water sample	25 ml
$K_2HPO_4$	0.02%
Ethanol	1.0% (or about 0.25 ml)
or	
Water sample	25 ml
$K_2HPO_4$	0.02%
Ethanol	1.0% (or about 0.25 ml)
Sodium benzoate	1.0%

The water samples are placed in 100-ml Erlenmeyer flasks.

Thompson (see Thompson and Skerman, 1979) isolated *A. insignis* from a rusty-brown, probably iron-oxide-rich water of a slow-flowing Australian creek with a pH of 6.7, using an enrichment medium recommended by V. Jensen (1955), by adding to the water sample 0.02%  $K_2HPO_4$  and 1% ethanol. The strain isolated (WR-51) was very similar to Derx's type strain as this strain also could not utilize glucose and excretes a violet diffusible pigment into the medium. In this respect it can be regarded as a neotype of Derx's original strain.

The acid tolerance of *Azomonas insignis* was also tested by V. Jensen (1955) of the other *Azomonas* and *Azotobacter* species. Most *A. insignis* strains could not grow in a medium with a pH lower than 5.7–5.9, although a few could produce faint growth at pH 5.5. Of the two *Azomonas agilis* strains tested, one could produce faint growth at pH 5.3, and one *Azotobacter beijerinckii* strain could even grow normally at pH 5.2. The one *Azotobacter chroococcum* strain tested in the survey could not grow below pH 6.1.

Other experiments also showed that *Azomonas insignis* strains have, in general, a preference for slightly alkaline conditions for optimal growth and nitrogen fixation. Of nine strains tested, three could grow at pH 6.0, but all strains grew at pH 10.0 (Thompson and Skerman, 1979). With respect to temperature requirements for growth, *A. insignis* strains seems to be able to grow at relatively lower temperature in comparison to other Azotobacteraceae. Nine of the 10 *A. insignis* strains tested by Thompson and Skerman (1979) could grow at 9°C, while the maximum growth temperature was 32°C.

A considerable reduction of the capacity to utilize carbon compounds by *A. insignis* compared to other *Azomonas* and *Azotobacter* species was first noted by V. Jensen (1955). Of the 26 compounds studied, *Azotobacter chroococcum* could utilize 16, *Azotobacter beijerinckii* on the average about 14, *Azomonas agilis* 11, and *Azomonas insignis* only 8. In a general survey, Thompson and Skerman (1979) tested 161 organic carbon compounds as sole source of carbon for *A. insignis*. Of these, 21 compounds could be utilized by nearly all *A. insignis* strains, 9 compounds could be utilized by a few strains, and no strain could utilize any of the 131 other organic carbon compounds tested.

### *Azomonas macrocytogenes*

In contrast to the two previous *Azomonas* species, *A. macrocytogenes* is isolated from soil. The species is primarily known from one isolate, called strain O (original), and two variants derived later, strains M (mutant) and I (intermediate). Although Thompson and Skerman (1979)

described seven strains, most of them are subcultures or derivatives of the original strains, and only one, a strain designated as the cotype of *Azotobacter agilis* subsp. *jakutiae* Krasil'nikov (1949), had another origin.

The original strain was isolated by H. L. Jensen (1955) in an attempt to isolate *Beijerinckia* from Danish soil. The isolate appeared on a nitrogen-free, mineral sucrose agar of about pH 5.5, seeded with garden soil (a fertile loam, pH 7.5) of the State Laboratory of Plant Culture, Lyngby, Denmark.

As could be anticipated from its isolation procedure, the type strain (type O and its variants) can produced good growth and nitrogen fixation in a nitrogen-free medium in the pH range of 4.6–6.9; one variant grew also at pH 4.3. This species is therefore even more acid tolerant than Tchan's (1953) and Jensen and Petersen's (1955) strains of *Azotobacter beijerinckii*.

Apart from morphological properties, such as ellipsoidal or rod-shaped cells, usually 2.5–3.5 µm in length and 1.6–2.1 µm wide, occurring singly or in pairs and occasionally forming short chains, and on media containing ethanol, the formation of very large coccoid, spindle-shaped or filamentous (up to 100 µm) cells, also physiological properties are important. The type strain showed no utilization of starch and rhamnose, but mannitol is utilized. Moreover, *A. macrocytogenes* strains can be differentiated from other *Azomonas* species by the utilization of mannitol (negative in *A. agilis* and *A. insignis*) and maltose (not utilized by *A. insignis* and most strains of *A. agilis*), and by its nonutilization of malonate (utilized by both *A. agilis* and *A. insignis*). In addition, *A. macrocytogenes*, being a soil organism, is more resistant to desiccation than *A. agilis* and *A. insignis*, which generally do not survive on silica gel for more than 2 days (Thompson and Skerman, 1979). However, no microcysts are formed, although microcyst-like structures occasionally are produced. These structures are more like capsules lacking the characteristic exine layer of a microcyst. Moreover, the cells sometimes have the tendency to become Gram-variable, i.e., to change from Gram-negative to Gram-positive.

The cells bear monotrichous flagella, but sometimes there are two flagella at one pole. Colonies do not produce a nondiffusible pigment, but on iron-deficient media, yellow-green or red-violet diffusible pigments are produced, which give a blue-white fluorescence in UV light.

In a numerical analysis and in the constructed dendrogram of relationships, Thompson and Skerman (1979) showed that *Azomonas macrocytogenes* fused at a low hierarchical level with *Azotobacter paspali*. At a higher level, the *Azomonas macrocytogenes*—*Azotobacter*

*paspali* group fused with the two other *Azomonas* species before fusing with *Azotobacter*. This was a motive for these authors to propose a new genus, *Azomonotrichon* (Thompson and Skerman 1979) for *Azomonas macrocytogenes*. However, (De Smedt et al. 1980) showed that the rRNA cistrons of *Azomonas insignis* and *A. agilis* differ as much from each other as they do from *Azomonas macrocytogenes* and from *Azotobacter*. Therefore, there is no reason to create a new genus for *Azomonas macrocytogenes*, although more genome comparisons of the various members of the Azotobacteraceae would probably be useful in clarifying its exact taxonomic position. It is noteworthy that Rubenchik (1959) transferred *Azomonas macrocytogenes* to the genus *Beijerinckia* as *B. macrocytogenes* because it fixed nitrogen at pH values of 4.5–5.0 and produced acid from certain carbohydrates. In support of this, Thompson and Skerman (1979) noted that *Azomonas macrocytogenes* produces colony-retained homopolysaccharides from sucrose and saccharose, and this is only found in this group and in most strains of the group containing the *Beijerinckia* species.

## Preservation of Cultures

For routine maintenance, *Azotobacter* and *Azomonas* cultures should be subcultured at monthly or bimonthly intervals on nitrogen-free mineral agar containing glucose or sucrose (1 or 2%). For those strains which cannot assimilate glucose (e.g., *Azomonas insignis*), another appropriate carbon source (e.g., an organic acid such as calcium malate or calcium succinate) can be chosen.

Poor results were obtained by lyophilization in skim milk or dextran-sodium glutamate solution on filter paper in small glass vials under vacuum with storage of the closed vials at room temperature in the dark. Antheunisse (1972, 1973) compared this method with storage of cultures on the usual agar media in normal culture tubes, plugged with sterile rubber seals, and stored in the dark at room temperature. Tests for viability of the cultures were made after periods of 1 to 10 years. The outcome showed that lyophilization of *Azotobacter* and *Azomonas* gave very poor results. Only 32% of the cultures were viable after 6 years. The rate of survival of *Azotobacter vinelandii* was relatively high, but that of *A. chroococcum* was low and that of *Azomonas agilis* was nil. In contrast, the 65 *Azotobacter* and *Azomonas* strains kept on sealed agar slants for 3–10 years gave an average viability of 60%. In this case, both *Azotobacter vinelandii* and *Azomonas agilis* gave good survival rates (86 and 78%, respectively).

In the author's laboratory, *Azotobacter* and *Azomonas* cultures are usually kept in normal culture tubes (with cotton plugs) under a seal of sterile liquid paraffin or mineral oil at room temperature (sometimes also at 4°C); such cultures generally survive for at least 3–5 years (J. H. Becking, unpublished observations). Care should be taken that the oil completely covers the agar slant, because if the agar medium remains in contact with air, the agar will dry out and the culture may die.

## Physiological and Biochemical Aspects

Azotobacteraceae are aerobic, heterotrophic, nitrogen-fixing organisms and therefore possess all oxidative enzymes for the degradation of the numerous organic carbon compounds utilizable by the various species as sole source of carbon and energy. Many of these enzymes are constitutive, but some are induced by the particular carbon substrate involved. Many publications have appeared on these enzymes, including some on the kinetics of their induction, beginning with the Wisconsin school (Stone and Wilson, 1952a, 1952b; Repaske and Wilson, 1953; Repaske, 1954; Williams and Wilson, 1954; Wilson and Wilson, 1955; Shutter and Wilson, 1955; Alexander and Wilson, 1956; Marr and Marcus, 1962), but they will not be discussed here in view of space limitations. Only some characteristic enzymes associated with nitrogen-fixation in Azotobacteraceae and the relation between the oxidative pathway and nitrogen fixation will be discussed.

The oxidative pathway in Azotobacteraceae as source of energy (ATP) follows the TCA cycle. It has been studied in many investigations (see above) using whole-cell suspensions or cell-free extracts to test the various intermediates of the TCA cycle, such as acetate, malate, succinate,  $\alpha$ -ketoglutarate, and citrate. Also, the effect of oxygen on nitrogen fixation and the high respiratory activity of Azotobacteraceae have received special attention, since reference is not an exact match Meyerhof and Burk (1928) and Burk (1930), first observed it, and it was the subject of many later studies (Tschapek and Giambiagi, 1955; Parker and Scutt, 1958, 1960; Dilworth and Parker, 1961). All the studies found that high oxygen levels, even atmospheric levels of oxygen, inhibited the nitrogen fixation activity in *Azotobacter*. Moreover, the exceptionally high respiratory quotients of *Azotobacter* species obtained by manometric methods were remarkable;  $Q_{O_2}$  values of 20,000  $\mu\text{l O}_2/\text{h}$  or 4,000–5,000  $\mu\text{l O}_2/\text{mg dry wt}/\text{h}$  have regularly been

measured (Williams and Wilson, 1954; Shutter and Wilson, 1955). Both observations have led to detailed investigations on the role of oxygen in the nitrogenase complex. Experiments revealed that in *Azotobacter* a special mechanism operates which protects the oxygen-sensitive nitrogenase from oxygen damage. The high respiration rate of Azotobacteraceae was explained as a mechanism which scavenges oxygen from the dinitrogen-fixing site of nitrogenase (Dalton and Postgate, 1969a). This process, called the "respiratory protection" of nitrogenase, is coupled with a multitude of cytochromes and redox proteins (Haddock and Jones, 1977), thus maintaining the nitrogenase in an essentially anoxic environment inside cells that nevertheless derive energy from aerobic metabolism.

The significance of respiratory protection has been argued, and alternative mechanisms have been suggested. A prominent argument was that the nitrogenase complex, which consists of two components (see later), was extremely sensitive to oxygen, being rapidly and irreversibly inactivated upon exposure to air, but nitrogenase in cell-free extracts of *Azotobacter* is relatively oxygen stable (Bulen et al., 1964; Kelly, 1969). The Brighton, England nitrogen-fixation school also suggested that if O<sub>2</sub> enters the cell more rapidly than it can be removed by respiration (i.e., by "respiratory protection"), an alternative control mechanism operates for which they proposed the name "conformational state protection," i.e., a protection by some steric arrangement of the components (Dalton and Postgate, 1969a). In the latter mechanism, during O<sub>2</sub> stress, nitrogenase binds to a 2Fe-2S protective protein, also called Fe/S II, to give an O<sub>2</sub>-stable complex that is protected from O<sub>2</sub> damage, but that is inert to nitrogenase substrates. When the oxygen stress is low or lowered by excessive respiration, the protected complex dissociates to give active nitrogenase. Such protective proteins have been isolated and characterized for *A. chroococcum* (Robson, 1979) and for *A. vinelandii* (Scherings et al., 1983).

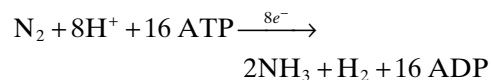
However, some alternative mechanisms have been proposed. Oppenheim et al. (1970a, 1970b) suggested that protection against oxygen was due to a major cytoplasmic membrane component that appears in these organisms when grown in nitrogen-free medium, while Yates (1970) suggested a form of respiratory control by nucleotides (ATP) operating in *Azotobacter*. Also, Kuhla and Oelze (1989) found a dependence of nitrogenase switch-off upon oxygen stress on the nitrogenase activity in *Azotobacter vinelandii*. Their results suggested that the flux of electrons to the nitrogenase complex, rather than cellular oxygen consumption (qO<sub>2</sub>), stabilizes nitrogenase activity

against O<sub>2</sub> inactivation in aerobically growing *A. vinelandii*.

From the observations mentioned above, it may be concluded that the mechanism of O<sub>2</sub> control and the absence of a serious inhibition of functioning nitrogenase by O<sub>2</sub> is a rather complex process, which may involve several different systems.

Although nitrogenase is the most important enzyme for aerobic nitrogen-fixing bacteria like the Azotobacteraceae, this enzyme is also found in a great number of other microorganisms belonging to quite-different taxonomic groups and affiliations. The main properties of nitrogenase (i.e., of the main species of nitrogenase, see later) can be summarized as follows: it consists of two proteins; it is sensitive to oxygen or it can be destroyed by oxygen; it contains the transition metals Fe and Mo; it needs Mg<sup>2+</sup> ions to be active; it converts ATP to ADP when functioning; it is inhibited by ADP; it reduces nitrogen and several other small triply bonded molecules (such as C<sub>2</sub>H<sub>2</sub> and CN); and it reduces H<sup>+</sup> ions to gaseous H<sub>2</sub> even when N<sub>2</sub> is present. The two proteins forming this nitrogenase are a large one (MW of about 220,000) and a smaller one (MW of about 60,000). Both proteins contain Fe (amounts variable), and this metal is essentially accompanied by S atoms. The large protein contains in addition two atoms of Mo and is therefore usually called the molybdoprotein. The proteins of the nitrogenase complex are often also called component I and component II. Furthermore, it is interesting that the two proteins are very similar in different nitrogen-fixing organisms. Their similarity is so great that it is possible to interchange these proteins with the proteins of other nitrogen-fixing microbes, regardless of their origin (e.g., *Klebsiella pneumoniae*, *Bacillus polymyxa*, and *Clostridium pasteurianum*) yielding fully active preparations (Bulen et al., 1966; Detroy et al., 1968; Dahlen et al., 1969).

Nitrogenase reduces N<sub>2</sub> to NH<sub>3</sub> and, for this function, it needs adenosine triphosphate (ATP). In vitro, 16 ATP molecules are consumed to convert one N<sub>2</sub> to two NH<sub>3</sub> molecules, and eight electrons are also involved:



Actually, the reduction of N<sub>2</sub> to two NH<sub>3</sub> requires six electrons, equivalent to 12 ATP molecules. It has been shown that two electrons or four ATPs are used for the formation of one molecule of H<sub>2</sub>. In growth experiments with *Azotobacter chroococcum* in continuous culture, Dalton and Postgate (1969b) demonstrated that nitrogen fixation entrained a maintenance coefficient of 1.06 g substrate/g organism/h compared with about



0.40 for ammonia assimilation. Assuming that most of this maintenance was directed to respiratory protection of nitrogenase, an extrapolated maximum requirement of 4 moles ATP/mole  $N_2$  fixed was observed.

In other growth experiments, Dalton and Postgate (1969a) observed that, at 0.03 atm  $O_2$ , nitrogen-limited, continuous cultures of *A. chroococcum* fixed about twice as much  $N_2$ /g carbon source utilized than at atmospheric pressure (i.e., 0.20 atm  $O_2$ ), confirming earlier growth experiments by other authors (Becking, 1971). The specific effects of different  $O_2$  levels on nitrogen-fixation efficiency of *Azotobacter* have already been discussed (see "General Identification").

As noted before, the nitrogenase system in *Azotobacter* consists of two nonheme iron proteins, i.e., a MoFe protein called Component I, which has been crystallized (Burns et al., 1970; Shah and Brill, 1973) and was shown to be a tetramer of 245,000 daltons (Swisher et al., 1977; Shah and Brill, 1977) containing two Mo atoms per molecule, and a protein called Component II, which is a Fe protein, a dimer of two identical subunits of 31,200 daltons, containing 289 amino acids (Hausinger and Howard, 1980). Thus, apart from Fe, the uptake of Mo by the organism is essential and a prerequisite for active functioning of the nitrogenase complex. Particularly, Mo deficiency or Mo depletion of the medium of Azotobacteraceae cultures limit their growth under nitrogen-fixing conditions. In growth experiments, it was shown by Becking (1962) that the Mo requirements of various *Azotobacter* and *Azomonas* species differ. Half-maximal nitrogen fixation was obtained in *A. chroococcum* at about 0.05 ppm Mo and in *A. vinelandii* at 0.0004 ppm Mo, whereas the same half-maximal growth value is reached in *Azomonas agilis* at 0.0002 ppm Mo. In these growth experiments, Becking (1962) further observed that in the majority of *Azotobacter chroococcum* and *A. vinelandii* strains, vanadium could replace molybdenum under nitrogen-fixing conditions. Of the 10 *A. chroococcum* strains tested, only three were unable to utilize vanadium as a substitute for molybdenum in nitrogenase and, in 19 out of the 20 *A. vinelandii* strains tested, vanadium was able to replace molybdenum in the nitrogen-fixation process. However, in all except one of 20 *Azomonas agilis* strains simultaneously tested, vanadium was unable to replace molybdenum in nitrogen fixation. Moreover, nitrogen fixation with vanadium was reduced to two-thirds of that produced by molybdenum. Experiments also demonstrated that for nitrate assimilation, molybdenum was also required in those species, which could assimilate nitrate (most *Azomonas agilis* strains are unable to utilize nitrate). But in

the nitrate reductase system, molybdenum could not be replaced by vanadium. Finally for ammonium assimilation, neither of the two metals is required (Becking, 1962).

In this context, it is of interest that an alternative nitrogenase system lacking molybdenum has been discovered and its existence proved biochemically and genetically. First evidence of such an alternative nitrogen-fixing system came from the genetic studies of Bishop et al. (1980, 1982), who obtained  $Nif^+$  pseudorevertants of the  $Nif^-$  strains UW6 and UW10 (see Shah et al., 1973) of *A. vinelandii*. These pseudorevertants displayed growth on nitrogen-free medium at a lower growth rate than the wild type and fixed nitrogen at a rate of 3–4% of the  $Nif^+$  control. Moreover, phenotypic reversion of  $Nif^-$  mutants to  $Nif^+$  occurred when they were grown on media lacking molybdenum but containing tungsten, vanadium, or rhenium salts (Bishop et al., 1980, 1982). Under these conditions, there was nitrogen fixation at a low rate although the cells lacked the typical EPR signal of the MoFe protein. These observations led Bishop et al. (1980, 1982, 1986) to propose an alternative pathway for nitrogen fixation in *A. vinelandii*, whose functioning was independent of molybdenum.

Following this, Eady et al. 1987 and Dilworth et al. 1988 demonstrated the existence of a vanadium nitrogenase in *A. chroococcum*. Miller and Eady (1988) showed that, for both the Mo and V nitrogenases present in *A. chroococcum*, low temperature favors the nitrogen reduction by the V nitrogenase. The vanadium nitrogenase of *A. chroococcum* was purified and the properties of the VFe protein were studied (Eady et al., 1987). These authors demonstrated that the VFe protein of the vanadium nitrogenase contained an iron-vanadium cofactor forming the substrate-reducing site (Smith et al., 1988). Also, the Fe protein of the vanadium nitrogenase was purified and characterized (Eady et al., 1988). Raina et al. 1988 characterized the gene for the iron-protein of the vanadium-dependent alternative nitrogenase of *A. vinelandii* and constructed a Tn5 mutant. Finally, the structural genes for the vanadium nitrogenase from *A. chroococcum* have been cloned and the nucleotides have been sequenced (Robson et al., 1989). There is also genetic evidence obtained using deletion mutant analysis that shows that at least in *Azotobacter vinelandii*, there is also a third nitrogenase that lacks both Mo and V (Pau et al., 1989). This has been independently verified, and the existence of an FeFe protein has been demonstrated (Smith, 1989; B.E. Smith, personal communication). Thus, in conclusion, three distinct nitrogenases are now known, each with its own, genetically distinct, Fe protein which acts as an electron transfer agent to either a MoFe, VFe, or FeFe

protein in an ATP-hydrolyzing reaction. The MoFe and VFe proteins have been shown to contain cofactors (FeMoco and FeVco) that form the substrate-reducing sites.

Besides oxygen, hydrogen has also been intensively studied in relation to nitrogenase (Robson and Postgate, 1980). Hydrogen evolution has received attention in the context of the efficiency of the nitrogen-fixing system (Schubert and Evans, 1976; Schubert et al., 1977). It was suggested that in a fully efficient system, all electrons would be used for ammonia production and no hydrogen would be evolved. All Azotobacteraceae have very powerful hydrogenases and therefore, in these organisms, the role of hydrogen in nitrogen fixation was of particular interest. Hydrogen-dependent mixotrophic growth of *A. vinelandii* has been observed (Wong and Maier, 1985). Moreover, hydrogen-mediated enhancement of hydrogenase expression (Prosser et al., 1988) and hydrogen-mediated mannose uptake (Maier and Prosser, 1988) have both been reported for *A. vinelandii*. Recently, competition studies in continuous culture between a Hup<sup>-</sup> (= Hydrogen-uptake<sup>-</sup>) mutant of *A. chroococcum* and its presumed isogenic Hup<sup>+</sup> recombinant showed that Hup activity benefitted the organism under nitrogen-fixing and sucrose- or phosphate-limiting conditions, but it was ineffective or disadvantageous under O<sub>2</sub>, sulfate, or iron limitation (Yates and Campbell, 1989). The physiological aspects as well as the genetics of the hydrogen-uptake hydrogenase has been lately reviewed by Yates (1988) and Yates et al. 1988.

With regard to the assimilation of combined nitrogen such as ammonia, the usual enzymes, including glutamate dehydrogenase (GDH), glutamine synthetase (GS), and glutamate synthase (GOGAT), have been reported for *A. chroococcum* (Drozd et al., 1972), and some have also been demonstrated in other *Azotobacter* species. GOGAT was found in both soluble and membrane-bound forms in *A. vinelandii*. Assimilatory nitrate and nitrite reductases have been characterized for *A. chroococcum* and *A. vinelandii* (Guerrero et al., 1973; Spencer et al., 1957; Taniguchi and Ohmachi, 1960; Vega et al., 1973).

## Genetic Aspects

The first metabolic mutants of *Azotobacter* were described for *Azotobacter vinelandii* by Karlsson and Barker (1948), and shortly afterwards mutants of the same species which do not fix nitrogen were reported by Wyss and Wyss (1950). The first DNA-mediated transformation

in *Azotobacter* was observed by Sen and Sen (1965), who described an interspecific transformation of pigment production between *A. chroococcum* and *A. vinelandii*. Transformation of Nif<sup>-</sup> strains of *A. vinelandii* with *Rhizobium* DNA have also been described (Page, 1977), together with some other intergeneric transformations between *Rhizobium* and *Azotobacter* (Sen et al., 1969; Bishop et al., 1977a; Maier et al., 1978). Although phages (azotophages) of *Azotobacter vinelandii* and *A. chroococcum* have been isolated (Monsour et al., 1955; Chumli et al., 1980), these phages had limited host ranges and apparently had no transducing ability (Bishop et al., 1977b). An earlier positive report (Wyss and Nimeck, 1962) mentioning interspecific transduction in *Azotobacter* could not be confirmed.

Most studies on mutants of *Azotobacter* have involved the *nif* genes. The first stable *nif*<sup>-</sup> mutants of *Azotobacter* were described for *A. vinelandii* by Fisher and Brill (1969). These mutants were biochemically analyzed by these authors for the activity of the two nitrogenase components (Shah et al., 1973; Bishop and Brill, 1977).

Regulatory mutants of *Azotobacter* that fix nitrogen in the presence of ammonium have also been isolated and described by Sorger (1968), Gordon and Brill (1972), Bishop and Brill (1977), and Terzaghi (1980b). Such mutants were found particularly among strains resistant to methylalanine. Ammonia-exporting mutants may be important in industry for ammonia production (see "Applications").

Bishop et al. 1980 obtained *nif*<sup>+</sup> pseudorevertants of some particular *nif*<sup>-</sup> mutants (UW 6 and UW 10), which could fix nitrogen at a rate of 2–4% of the *nif*<sup>+</sup> control. From these observations, they inferred the existence of an alternative pathway for nitrogen fixation functioning independently of Mo (see "Physiological and Biochemical Aspects"). Moreover, drug-resistant mutants (Page and Sadoff, 1976; Bishop and Brill, 1977) and amino acid and vitamin autotrophs (Leach and Battikhi, 1978) of *A. vinelandii* have regularly been obtained by mutagenesis and the application of selective media.

On the whole, *Azotobacter* species and, in particular, *Azomonas* species seem to be difficult to mutate or the selection procedures used so far have been inadequate. Sadoff et al. 1979 argued that *A. vinelandii* contains 40 chromosomes per cell, and therefore failure to isolate mutants could result from difficulties in segregation rather than mutagenesis. In view of these difficulties, most information on the *nif* genes comes from *Klebsiella pneumoniae*. It has a good transduction system and a plasmid (pRD1) that carries the entire *nif* cluster of *K. pneumoniae*. The plasmid has been isolated and this system was

most useful in the development of the *Azotobacter nif* genetics, e.g., by the expression of *Klebsiella nif* genes in *Azotobacter* (Cannon and Postgate, 1976).

At the moment, the homology of *nif* genes of *Azotobacter* and other bacteria species is well established, and the genes encoding the two nitrogenase systems in two species (*A. vinelandii* and *A. chroococcum*) have been identified.

A group of genes spanning 25–30 kb of DNA was characterized in *A. chroococcum* after being cloned and their expression studied in *Klebsiella pneumoniae* (Jones et al., 1984). Hybridization to *nif* gene probes from *K. pneumoniae*, coupled with DNA sequencing and complementation analysis, revealed that the *nif* genes *FMV-SUNEKDH* are present in this region (Evans et al., 1985). The genes best characterized for structure and function in *A. vinelandii* and *A. chroococcum* are *nifKDH*. A restriction map for a region of the *A. chroococcum* genome carrying *nif* genes (Jones et al., 1984) confirms close linkage and expression of *nifKDH* as a single transcript, and places *nifV* 15 kb away from *nifKDH*.

The promoters of *nif* genes were observed to share common nucleotide sequences in two regions upstream from the site at which transcription begins in a number of different organisms such as *Azotobacter*, *Klebsiella*, *Rhizobium*, *Thiobacillus*, and *Desulfovibrio*. The structural genes, including that of the VFe protein of the vanadium nitrogenase from *A. chroococcum* (Robson et al., 1989), have been cloned, and nucleotide sequences were determined as already reported. Activator proteins are needed for *nif* gene expression, and genes encoding activator genes have diverged to control expression of the three different enzyme systems responsible for nitrogen fixation. Regulation of *nif* genes in *Azotobacter* is therefore rather complicated.

Similarity of *nif* regulation among *K. pneumoniae* and *A. vinelandii* and *A. chroococcum* has been demonstrated (Kennedy and Robson, 1983). Introduction of the *nifA* gene from *K. pneumoniae* into *Azotobacter* mutants deficient in both components I and II restored the Nif<sup>+</sup> phenotype to the presumed regulatory mutants but not to a nitrogenase-structural-gene mutant. The results are interpreted to suggest that *nifA* activation of *nif* genes might also be conserved among diazotrophs. Two nitrogen fixation regulatory regions, *nifA* and *nfrX*, in *Azotobacter vinelandii* and *A. chroococcum* have also been identified and characterized (Santero et al., 1988). These investigations are too numerous to cite here fully, but most of them are cited in recent reviews on the genetics of *Azotobacter* and related organisms by Elmerich (1984), Kennedy and Toukdarian (1987), and Kennedy (1989).

## Applications

Associative growth of one *Azotobacter* species, *A. paspali*, has been reported to produce growth responses, apparently giving an increase in nitrogen, in forage grasses (Döbereiner, 1970; Döbereiner and Day, 1976).

However, *Azotobacter* inoculants (mainly *A. chroococcum*) also may produce crop responses. Especially in the USSR in the years 1958–1960, numerous field experiments were conducted with *Azotobacter* inoculants (named “Azotobacterin” or “Nitragin”) with agricultural crops like spring wheat, winter wheat, barley, oats, and maize. Mishustin and Shilnikova (1969, 1971) have summarized these results, showing that in some trials, significant yield increases were obtained with a beneficial effect varying from 7–12%. Later, however, this practice was no longer recommended and it has now been abandoned.

However, some workers still claim that *Azotobacter* has a positive effect on crops, e.g., *Azotobacter* inoculation of seeds or seedlings of wheat, rice, onion, tomato, brinjal (*Solanum aestivum*), and cabbage. Significant responses with increases that average 10–20% are reported for such crops (Sundara Rao et al., 1963; Lehri and Mehrotra, 1968, 1972; Mehrotra and Lehri, 1971; Joi and Shinde, 1976; Shende et al., 1977).

Some other workers (Vančura and Macura, 1959, 1961; Brown et al., 1962, 1964) performed field and pot experiments in which artificial inoculation produces increases in the yield of crops. They attributed the beneficial responses in addition to fixed nitrogen made available to the plants by the production of phytohormones such as gibberellins, auxins, and some phenolic compounds (Jackson et al., 1964; Hennequin and Blachère, 1966), giving the plants a better health condition. Also, the production of antifungal antibiotics by *Azotobacter* may play a rôle in these yield increases (Mishustin and Shilnikova, 1969, 1971; reference is not an exact match Laksmi Kumari et al., 1975).

For instance, Meshram and Jager (1983) mentioned an antagonism of isolates of *Azotobacter chroococcum* to *Rhizoctonia solani* on agar plates and *Azotobacter* isolates were tested for their ability to control *R. solani* infection of potato sprouts in sterilized and unsterilized soil. The degree of antagonism exhibited varied strongly among the isolates and was found to be temperature-dependent. Following this, Azad and Aslam (1985) observed that *Azotobacter chroococcum* inoculation increased the yield and the protein content of potato (*Solanum tuberosum*) tubers. Analyses showed that the inoculum increased substantially the population of *A. chroococcum* present in the rhizosphere soil in these experiments.

Bagyaraj and Menge (1978) showed that larger populations of bacteria and actinomycetes were recovered from the rhizospheres of tomato (*Lycopersicon esculentum*) plants inoculated with the mycorrhizal fungus *Glomus fasciculatus* and *Azotobacter chroococcum*, either individually or together, than from those of non-inoculated plants. The dry weights of the tomato plants inoculated with both *G. fasciculatus* and *A. chroococcum* were significantly (62%) greater than non-inoculated plants. These results suggest a synergistic or additive interaction between *Glomus fasciculatus* and *Azotobacter chroococcum*.

Although the practice of using *Azotobacter* inoculants was often unsatisfactory and has been abandoned in many countries, in India a constant stream of publications continues to appear on the beneficial effects of *Azotobacter* on various crops such as sorghum, pearl millet, maize, rice, sesame, wheat, and barley (Ghonsikar et al., 1986; Prasad, 1986; Konde and Shinde, 1986; Shende et al., 1986; Subbian and Chamy, 1984; Rai and Gaur, 1988; Kavimandan et al., 1978; Tiwari et al., 1989). In India, it is also suggested that *Azotobacter* inoculation gives positive growth responses in oak seedlings used in forestry (Pandey et al., 1986).

Outside India, there are now only sporadic reports on the beneficial effects of *Azotobacter* inoculations, and these refer mainly to subtropical regions. Some Egyptian authors have obtained positive results on the growth of the castor oil plant, *Ricinus communis* (Monib et al., 1984), or of wheat (Emam et al., 1986) with *Azotobacter* inoculation.

Monib et al. 1979 also studied the effect of bacterization of barley (*Hordium vulgare*) grains with a selected strain of *Azotobacter chroococcum*. In N-deficient sand, seed inoculation increased plant length, dry weight, nitrogen content in addition to a significant increase in soil nitrogen. In the presence of a mixed soil microflora the beneficial effect of bacterization was less than in monobacterial cultures. *Azotobacter* naturally present in soil also colonized in heavy densities the rhizosphere (rhizosphere) of the barley plants, but their effect on plant growth and soil nitrogen were less as compared with that of bacterization.

In Spain, the beneficial effects of *Azotobacter chroococcum* on root colonization and grain production of maize have been reported (Martinez-Toledo et al., 1988a, 1988b), and in *Sorghum bicolor*, a specific root association with *A. chroococcum* was demonstrated, but there was no interaction influencing the nitrogenase activity in the rhizosphere, probably due to the lack of excretion of a carbon and energy source by the plant (De La Rubia et al., 1989). In addition, in

Israel, positive responses of *Azotobacter* inoculation on the growth of *Setaria italica* have been reported (Yahalom et al., 1984). In Pakistan, Hussain et al. 1985 studied in field experiments the seed inoculation of wheat (*Triticum aestivum*) on the yield in relation to the application of farm yard manure and some N-P-K levels, in which N was given in the form of urea. The results indicated that *Azotobacter* inoculation was more effective as regards to grain yield in the trials where no nitrogen was added, next was farm yard manure and the lowest response was with urea application.

From all these investigations it is clear that, with regard to beneficial effects, the supply of fixed N might not be the sole factor involved or it is only a minor factor and that growth-promoting substances may be involved. In particular, it appears that plant root colonization by bacteria is favorable, probably due to biological control of plant diseases (see e.g., Kloepper et al., 1989).

Some *Azotobacter* mutants may be important for biotechnology and industrial application. Mutants of *A. vinelandii* have been obtained that fix molecular nitrogen in the presence of excess  $\text{NH}_4^+$  excretion into the medium (Gordon and Brill, 1972; Shaw et al., 1973). Terzaghi (1980a, 1980b) tried to produce such mutants in a variety of *Azotobacter*/*Azomonas* species, i.e., mutants which could excrete excess ammonia in addition to being able to reduce  $\text{N}_2$  in the presence of excess  $\text{NH}_4^+$ . She isolated nitrogenase-derepressed (Nif-Drd) mutants of *Azotobacter* in the two *Azotobacter* species (*A. vinelandii* and *A. beijerinckii*) and the two *Azomonas* species (*A. agilis* and *A. macrocytogenes*) tested. Such mutants could only be obtained in *Azotobacter vinelandii* and to a much lesser degree in one strain of *Azomonas agilis*. These mutants, having up to 100% nitrogenase activity and excreting  $\text{NH}_4^+$  into the medium without inhibiting the nitrogenase activity, are of industrial significance, for instance by immobilization in Alginate beads, because they offer the opportunity to produce ammonia (which can be used as plant fertilizer) using *Azotobacter* in a biotechnological process.

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## The Genera *Beggiatoa* and *Thioploca*

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### Introduction

Filamentous sulfur-oxidizing bacteria of the genera *Beggiatoa* and *Thioploca* are some of the largest and most conspicuous bacteria in nature (Schulz and Jørgensen, 2001). Their white or yellow color, their filamentous morphology, the large width and length of their filaments, and their growth pattern in flocs and mats on sediment surfaces makes them highly conspicuous even to the unaided eye. The two genera are distinguished by a single morphological character: *Thioploca* filaments occur in bundles surrounded by a common sheath, whereas *Beggiatoa* filaments do not form this structure and occur as individual filaments. All *Beggiatoa* and *Thioploca* strains have the ability to oxidize sulfide to elemental sulfur that is stored as intracellular sulfur globules, which make the cells highly refractory and conspicuous under the microscope. This characteristic, together with the absence of photosynthetic pigments, has distinguished the genera *Beggiatoa* and *Thioploca* as filamentous members of the “colorless sulfur bacteria” from other filamentous bacteria, for example the cyanobacteria or nonsulfur-oxidizing heterotrophs such as *Cytophaga* and *Flexibacter*. Contrary to earlier assumptions (Reichenbach and Dworkin, 1981), there is no close evolutionary relationship between other gliding filamentous bacteria, and *Beggiatoa* and *Thioploca*.

The metabolic spectrum in *Beggiatoa* ranges from obligate autotrophy with sulfide and other reduced sulfur compounds as energy source, so far found in marine strains only (Nelson and Jannasch, 1983), to seemingly strict heterotrophy based on organic carbon and energy sources (Nelson and Castenholz, 1981a). The *Thioploca* spp. that have been characterized so far, have autotrophic potential (Otte et al., 1999). Biogenic or geothermal sulfide is oxidized to elemental sulfur, and subsequently in autotrophic strains to sulfate, to provide energy and reducing power for CO<sub>2</sub> fixation and biomass synthesis. The electron acceptor for of sulfide oxidation is oxygen (Nelson et al., 1986b) or in some cases

nitrate (McHatton et al., 1996; Otte et al., 1999). The requirement for oxygen and sulfide, two mutually highly reactive chemical species, forces *Beggiatoa* and *Thioploca* into a specialized niche at naturally occurring oxic/anoxic interfaces where these bacteria compete efficiently with chemical sulfide oxidation (Jørgensen, 1982). *Beggiatoa* grows at oxic/anoxic interfaces, where sulfide as electron donor and oxygen as electron acceptor are present simultaneously (Nelson et al., 1986b). Large marine *Beggiatoa* also adapt to fluctuating gradients by increased intracellular storage capacity for oxidants (McHatton et al., 1996). *Thioploca* filaments can bridge spatially separated pools of oxidant and reductant by moving up and down and adjusting their position in a sediment-embedded sheath (Hüttel et al., 1996). *Beggiatoa* and *Thioploca* are effective mat-forming microorganisms; their mats may be less than a millimeter thin (Nelson et al., 1986a) or several centimeters thick (Jannasch et al., 1989b), depending on the dynamics and stability of the sulfide/oxidant interface. The most extensive microbial mats on earth are formed by *Thioploca* on the Chilean and Peruvian continental shelf (Gallardo, 1977) and are of considerable importance as a link of the marine carbon, sulfur and nitrogen cycles (Fossing et al., 1995; Jørgensen and Gallardo, 1999).

The filamentous sulfur-oxidizing bacteria play a special role in the history of microbiology. The concept of chemolithoautotrophy, light-independent biomass production by CO<sub>2</sub> fixation and oxidation of inorganic electron donors, originated with Winogradsky's studies of *Beggiatoa* enrichment cultures (Winogradsky, 1887). Winogradsky described lithotrophy but not autotrophy in this pioneering work, and a full understanding of the concept we now call lithoautotrophy arose from his later pure culture studies of nitrifying bacteria (Brock and Schlegel, 1989). After a protracted search for *Beggiatoa* strains that could grow autotrophically on reduced sulfur compounds and CO<sub>2</sub> without any addition of organic substrates (briefly outlined in Nelson and Castenholz, 1981b), autotrophic marine *Beggiatoa* were isolated in pure culture

and characterized in detail a century after Winogradsky's pioneering work (Nelson and Jannasch, 1983; Hagen and Nelson, 1996). In nature, the autotrophic biomass production of *Beggiatoa* mats and other sulfur-oxidizing bacteria represents "primary production" if the source of sulfide is geothermal, or "secondary production" if it recaptures some of the energy of anaerobic carbon remineralization via sulfate reduction that produces sulfide (Jannasch, 1989a).

## Habitats

The filamentous sulfur oxidizers *Beggiatoa* and *Thioploca* grow at oxic/anoxic interfaces on freshwater, brackish and marine sediments where sulfide of biological or geothermal origin comes in contact with oxygen or nitrate in the overlying water column. *Beggiatoa* species rely on the simultaneous presence of sulfide and oxidant in steep, narrowly overlapping, opposing gradients (Jørgensen and Revsbech, 1983a; Nelson et al., 1986b) or are adapted to alternating exposure to sulfide and oxidant in frequently fluctuating water flow (Gundersen et al., 1992). *Thioploca* filaments have the ability to adjust their position in redox gradients by emerging from, retreating into, and moving up and down within their sediment-embedded sheaths (Hüttel et al., 1996). This ecophysiological flexibility allows *Beggiatoa* and *Thioploca* to colonize a wide spectrum of freshwater and marine environments. Typical freshwater *Beggiatoa* habitats are sulfur springs (Uphof, 1927; Caldwell et al., 1975; Nelson and Castenholz, 1981a; Fukui et al., 1999), ditches, puddles, wetlands, and lake sediments (Scotten and Stokes, 1962; Pringsheim, 1964; Strohl and Larkin, 1978; Fig. 1). In rice paddies that experience sulfate-enrichment, *Beggiatoa* grows in association with rice roots (Pitts et al., 1972; Joshi and Hollis, 1977). In the marine



Fig. 1. Freshwater *Beggiatoa* mat in Duckstein Spring, Germany. Courtesy of Manabu Fukui.

environment, *Beggiatoa* can be found in a wide range of habitats, including organic-rich, coastal marine sediments (Jørgensen, 1977; Mussmann et al., 2003), salt marshes (Nelson et al., 1982b; Fig. 2), naturally eutrophic, oxygen-depleted bays (Graco et al., 2001), oxygen-depleted marine basins (Williams and Reimers, 1983; Kuever et al. 1996), geothermally active submarine caves (Mattison et al., 1998), hydrothermal vents (Jannasch et al., 1989b; Nelson et al., 1989c; Fig. 3), cold sulfide seeps (Sassen et al., 1993), hydrocarbon seeps (Larkin et al., 1994), and sulfidic sediments overlying gas hydrates (Sahling et al. 2002). Highly localized areas of anaerobic decomposition, such as a whale carcass on the ocean floor, also lead to enrichment of *Beggiatoa* spp. (Smith et al., 1989).

In general, *Beggiatoa* spp. prefer a microoxic habitat at the interface of a source of reduced sulfur, typically sulfide, and oxygen or nitrate as electron acceptors. Detailed microelectrode surveys with non-vacuolate *Beggiatoa* species in gradient cultures showed that oxygen-respiring

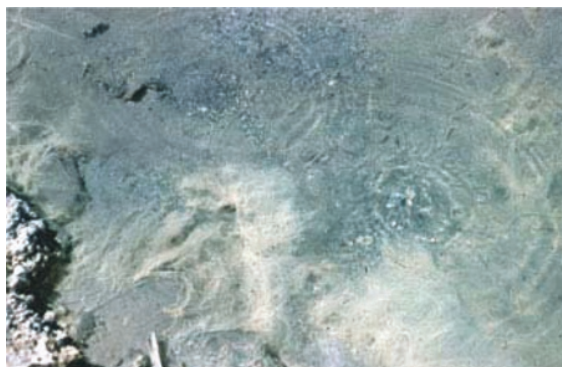


Fig. 2. White, streaming *Beggiatoa* filaments in Sippewissett salt marsh. Courtesy of Holger Jannasch.

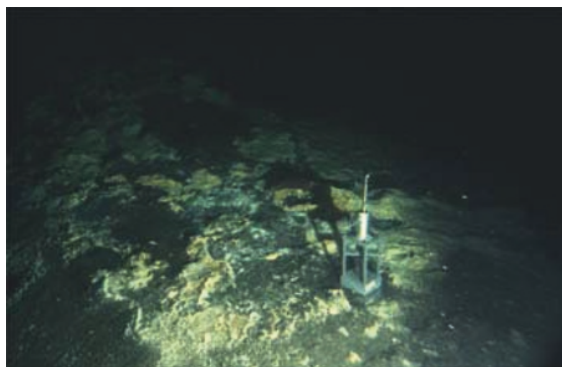


Fig. 3. Guaymas *Beggiatoa* mats. Yellow-orange *Beggiatoa* mats on hydrothermally flushed sediments of the Guaymas Basin. The in situ incubation device in the foreground is ca. 50 cm high. Courtesy of Holger Jannasch.

*Beggiatoa* grows at oxygen partial pressures of <6% air saturation (Nelson et al., 1986a; Nelson et al., 1986b). Similarly, low oxygen partial pressures have been found in *Beggiatoa* mats in nature, on organic-rich marine sediments (Fenchel and Bernard, 1995). In photosynthetic cyanobacterial mats, *Beggiatoa* filaments constitute a part of the mat matrix and follow the diurnally changing sulfide-oxygen interface (Garcia-Pichel et al., 1994). Large marine *Beggiatoa*, which most likely depend on nitrate as electron acceptor (McHatton et al., 1996), are found growing in dense mats at nitrate-rich, oxygen-poor sediment-water interfaces. These large *Beggiatoa* reach filament diameters of up to 200  $\mu\text{m}$  and grow in patchy mats on the surface of hydrothermal vent sediments and of cold-seeps; they are among the largest known prokaryotes (Larkin and Henk, 1996). Interestingly, large *Beggiatoa* spp. growing in land-locked saline springs often resemble their counterparts in the marine environment and share the conspicuous vacuolated phenotype that is typical of many marine populations (Kolkwitz, 1918).

*Thioploca* forms ensheathed bundles of filaments that can extend and retreat into the sediment, several cm in large marine species, although the bulk of the biomass is still found near the sediment surface (Schulz et al., 1996; Schulz et al., 2000). With the discovery of extensive marine mats of large *Thioploca*, this bacterium emerged as a significant microbial component of the sulfur and nitrogen cycles in the oceans (Fossing et al., 1995). Extensive mats of large *Thioploca* were found on the Chilean and Peruvian continental shelf, where they grow on sediments that underlie the deoxygenated water masses of the Peru-Chile countercurrent (Gallardo, 1963; Gallardo, 1977). Bundles of a few or up to one hundred *Thioploca* filaments are surrounded by a polysaccharide sheath (Fig. 4). A mesh of these conspicuous, ensheathed whitish bundles, each up to several centimeters long and one millimeter thick, is embedded in soft, organic-rich sediments and forms a mat of several cm thickness (Fig. 5). In its entirety, this *Thioploca* habitat stretches—with considerable patchiness and seasonal variability—from the continental shelf of south-central Chile to Peru (Gallardo, 1975; Rosenberg et al., 1983; Zafra et al., 1988; Gallardo et al., 1995; Carrasco et al., 1999), over a distance of 3000 km, and constitutes the most extensive microbial mat habitat on Earth (Jørgensen and Gallardo, 1999). *Thioploca* has been found in coastal regions with similar upwelling regimes, where high organic productivity causes significant oxygen depletion at the bottom waters that overlay organic-rich sediments with high sulfate reduction rates. Examples include the coast of

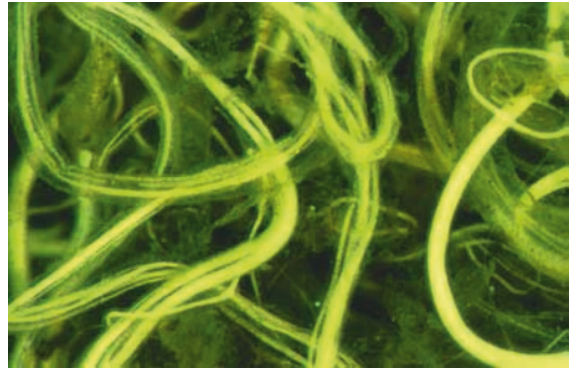


Fig. 4. Rinsed *Thioploca* bundles from the Chilean Coast. The gelatinous, transparent sheaths harbor white *Thioploca* filaments. The larger filaments of the species *Thioploca araucae* have a diameter of ca. 40  $\mu\text{m}$ . Courtesy of Markus Hüttel.

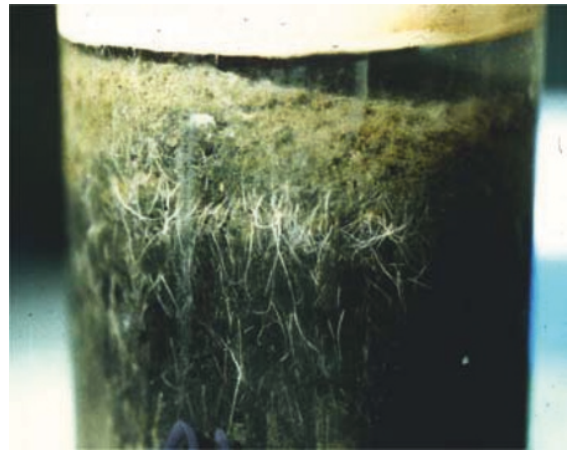


Fig. 5. Gravity core with sediments harboring *Thioploca* mat, from the Chilean Pacific shelf near Concepción. Individual white *Thioploca* filaments extend several cm into the dark brown sediment. Most of the *Thioploca* biomass is found near the sediment surface where *Thioploca* filaments form a dense web. Courtesy of Markus Hüttel.

Oman (Schmaljohann et al., 2001), and the Benguela current ecosystem off Namibia (Gallardo et al., 1998). Early studies of *Thioploca* were based on freshwater and brackish water habitats, for example in fresh water sediments of Lake Constance (Lauterborn, 1907), the Neva river at St. Petersburg (Wislouch, 1912), in the Rhine and in Baltic coastal lagoons (Kolkwitz, 1912), and in Lake Erie (Maier, 1980). Many of these small-scale habitats are affected by environmental change and human impact, with the result that many older sampling sites do not exist today (Maier and Preissner, 1979). Recently, mats of freshwater *Thioploca* spp. have been found in Lake Baikal in Siberia (Namsaraev et al., 1994; Zemskaia et al., 2001), in Lake Ontario between the United States and Canada (Dermott and

Legner, 2002) and in Lake Biwa, Japan (Nishino et al., 1998; Kojima et al., 2003). These extensive freshwater *Thioploca* mats offer the opportunity to study freshwater *Thioploca* spp. in similar detail as their marine counterparts.

## Identification and Taxonomy

The genera *Beggiatoa* and *Thioploca* are recognized primarily on the basis of their filamentous morphology and, in the case of *Thioploca*, their growth pattern as bundles of filaments surrounded by a gelatinous sheath. For *Beggiatoa*, the filament lengths are from a few  $\mu\text{m}$  to 10 cm; the cell and filament diameter range between ca. 1–200  $\mu\text{m}$ . For *Thioploca*, filaments reach 2–5 cm in length, and reach diameters of 2.5–80  $\mu\text{m}$ . Another reliable characteristic is the ability to oxidize sulfide and to store the intermediate oxidation product, elemental sulfur, in small intracellular, highly refractory and conspicuous sulfur globules. This character separates the genus *Beggiatoa* from the filamentous genus *Vitreoscilla*, which is obligately heterotrophic and does not form intracellular sulfur globules by sulfide oxidation. The genus *Beggiatoa* differs from the filamentous genus *Thiothrix* by the specific attachment of the latter via specialized “hold-fast” cells (often in a rosetta-like growth pattern) of the latter, and is also phylogenetically distinct (Teske et al., 1995; Howarth et al., 1999).

The genera *Beggiatoa* and *Thioploca* harbor considerable morphological and physiological diversity that is not adequately represented by the small number of recognized species. Within the genus *Beggiatoa*, three major phenotypically defined groups can be distinguished: 1) the heterotrophic nonvacuolate freshwater strains with thin filament diameter, represented by several well-studied strains: The type strain of the genus *Beggiatoa* (B18LD; Mezzino et al., 1984), B15LB (Strohl et al., 1982), OH-75-2a (Nelson and Castenholz, 1981b; Nelson and Castenholz, 1981a), and L1401-13 (Pringsheim, 1964; Kowallik and Pringsheim, 1966; Fig. 6); 2) the autotrophic nonvacuolate marine strains with thin filament diameter, represented by the facultatively autotrophic strain MS-81-6 and by the obligately autotrophic strains MS-81-1c (Nelson et al., 1982b; Nelson et al., 1986a; Nelson and Jannasch, 1983; Hagen and Nelson, 1996; Hagen and Nelson, 1997; Fig. 7); and 3) the large, vacuolated, nitrate-accumulating autotrophic marine *Beggiatoa* strains which so far remain uncultured (Hinze, 1901; Jannasch et al., 1989b; Nelson et al., 1989c; McHatton et al., 1996; Fig. 8).

In contrast to *Beggiatoa* filaments, *Thioploca* filaments aggregate into bundles that are enclosed by a robust polysaccharide sheath,

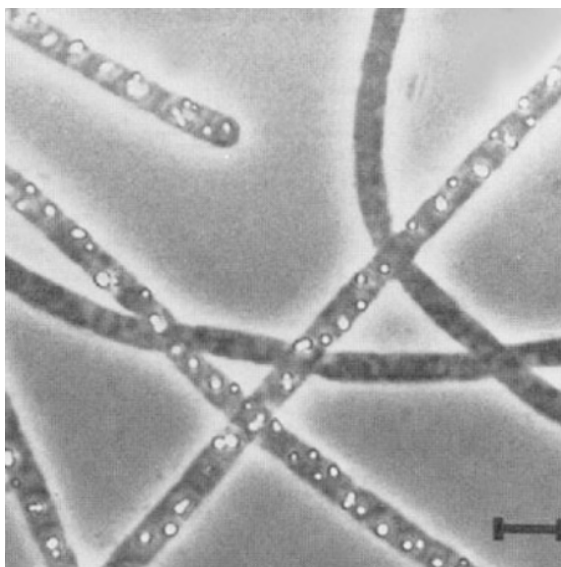


Fig. 6. Light micrograph of a pure culture of freshwater strain OH-75-2a, stationary phase, grown in liquid DTA medium. Some filaments are devoid of refractile S<sub>0</sub> globules. Nonrefractile inclusions are probably poly- $\beta$ -hydroxybutyrate (PHB) granules. Bar = 5  $\mu\text{m}$ . Phase contrast. Courtesy of Douglas Nelson.

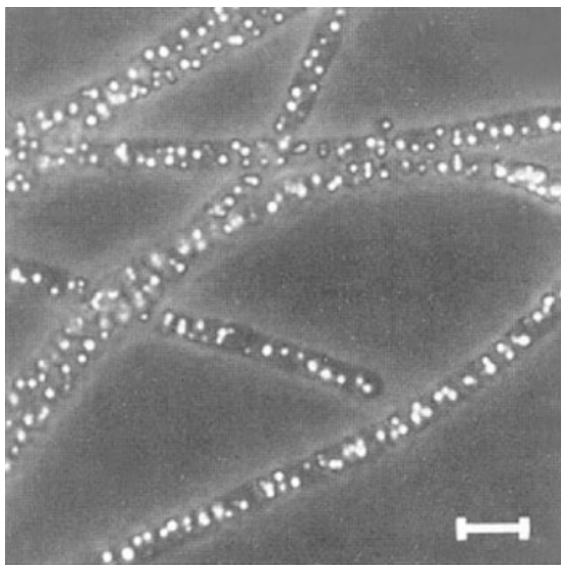


Fig. 7. Light micrograph of a pure culture of marine strain MS-81-1c during chemoautotrophic growth in gradient medium. Note the refractile S<sub>0</sub> globules. Bar = 5  $\mu\text{m}$ . Phase contrast. Courtesy of Douglas Nelson.

the defining character of the genus *Thioploca* (Maier, 1989). The ensheathed bundles, which contain a few to a few dozen of these filaments, occur mostly embedded in sediments and plant detritus; they can reach lengths of several centimeters in large *Thioploca* species and thus form

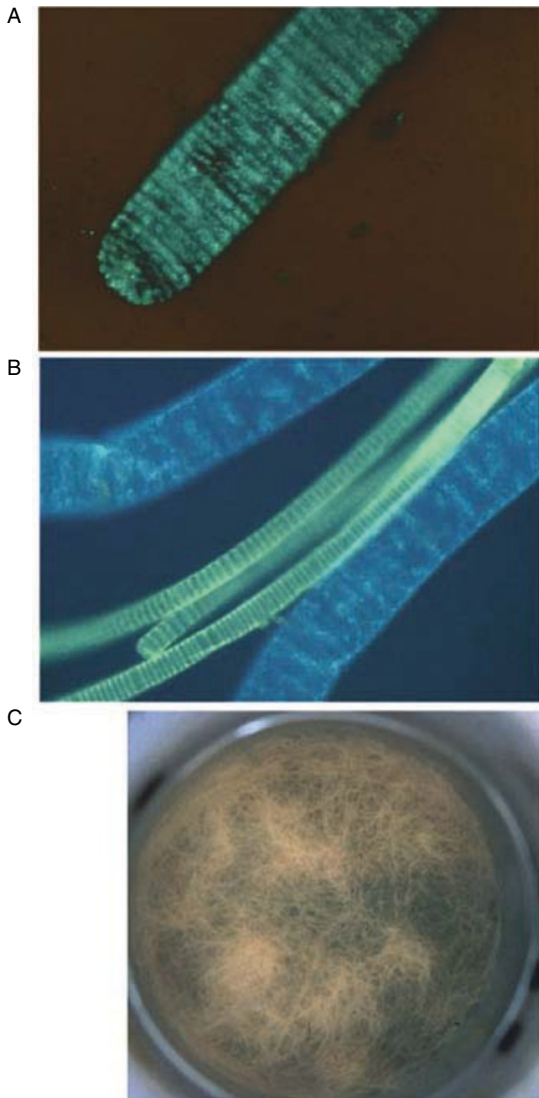


Fig. 8. Large marine *Beggiatoa* spp. from the Guaymas Basin and Monterey Bay. A) Filament tip of a very large, nitrate-accumulating *Beggiatoa* from the Guaymas Basin hydrothermal vents. The filament is ca. 160  $\mu\text{m}$  in diameter. The highly refractory sulfur globules outline the disk-like cells within the filament. Dark field microphotograph. B) Two *Beggiatoa* spp. from Guaymas under ultraviolet (UV) light. The larger species (ca. 100  $\mu\text{m}$  filament diameter) is fluorescing blue, the smaller species (ca. 35  $\mu\text{m}$  filament diameter) yellow-orange. UV epifluorescence microphotograph. Courtesy of Andreas Teske. C) Large *Beggiatoa* spp. (65–85  $\mu\text{m}$  filament diameter) from Monterey Canyon mat, on sediment core. Courtesy of Douglas Nelson.

one of the largest prokaryotic structures. These bundles are conspicuous not only by their large size, but also by their whitish or beige color, and have indeed reminded several investigators of slightly overcooked angel hair pasta, leading to the nickname “spaghetti bacteria.” In the genus *Thioploca*, no cultured species exist. Here, at

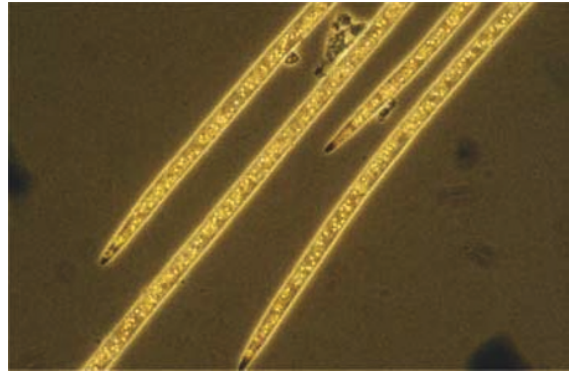


Fig. 9. Microphotograph of *Thioploca ingrica*. Sulfur-rich *Thioploca ingrica* filaments and filament tips. Specimen from decaying reed in Randersfjord, Denmark. Filament diameter is ca. 4  $\mu\text{m}$ . Courtesy of Andreas Teske.

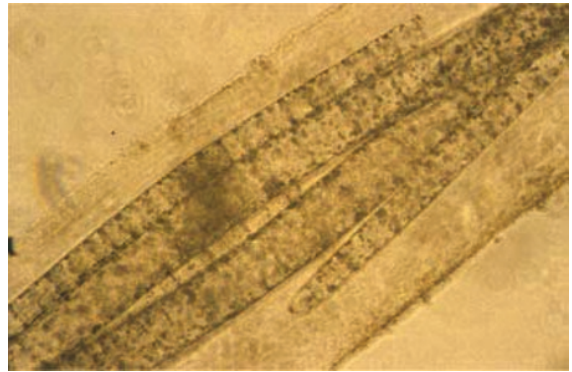


Fig. 10. Microphotographs of *Thioploca chileae* and *T. araucae*. Filaments of *Thioploca chileae* and *Thioploca araucae* in the same sheath. Filament diameters 18–20  $\mu\text{m}$  (*T. ingrica*) and 31–35  $\mu\text{m}$  (*T. araucae*). Specimen collected from the Chilean Pacific Shelf near Concepción. Courtesy of Andreas Teske.

least two phenotypic groups can be distinguished: 1) The freshwater or brackish *Thioploca* spp. with thin filament diameter and no visible or only modest vacuolation (Lauterborn, 1907; Wislouch, 1912; Maier and Murray, 1965; Fig. 9); and 2) the large, highly-vacuolated, nitrate-accumulating autotrophic marine species *Thioploca chileae* and *Thioploca araucae* (Maier and Gallardo, 1984; Fig. 10).

For both *Beggiatoa* and *Thioploca*, these classifications almost certainly hide as much diversity as they reveal and should not be regarded as complete but rather represent working definitions on the basis of current insufficient knowledge. Exceptions and borderline cases exist, such as the recent demonstration that an autotrophic freshwater *Beggiatoa* sp. does exist (Grabovich et al., 1998; Grabovich et al., 2001; Patrinskaya et al., 2001), and the observation that *Thioploca ingrica* has cytoplasmic vacuolation. Although

examination of specimens from Danish fjords did not reveal nitrate accumulation (L. P. Nielsen, personal communication), high intracellular nitrate accumulation, by four orders of magnitude, has been found in small freshwater *Thioploca* spp. (2–5 µm diameter) from underwater thermal vents in Lake Baikal (Zemskaya et al., 2001).

Further, the generalization that filamentous morphology prevails in these groups has exceptions. *Thiomargarita namibiensis*, a close phylogenetic relative of the large marine *Thioploca* species, occurs as a series of large, balloon-like cells that are lined up in short chains within a shared sheath, or may exist as individual cells, but not as filaments (Schulz et al., 1999; Fig. 11).

*Thioploca* species have been described solely based on filament diameter of consistently recurring size classes of specimens collected in the environment (Table 1); these data were later complemented by 16S rRNA sequences. The identification and recognition of species based on morphological and genotypic characteristics of specimens collected from the environment is unusual, but this system has been applied in several cases to morphologically conspicuous microorganisms that can be reproducibly obtained from natural source materials (*Epulopiscium*

*fishelsoni*, *Thiomargarita namibiensis*, *Thiovulum majus*, and *Achromatium oxaliferum*). For *Beggiatoa*, the type species *Beggiatoa alba*, a freshwater, non-autotrophic isolate, remains so far the only recognized species (Mezzino et al.,

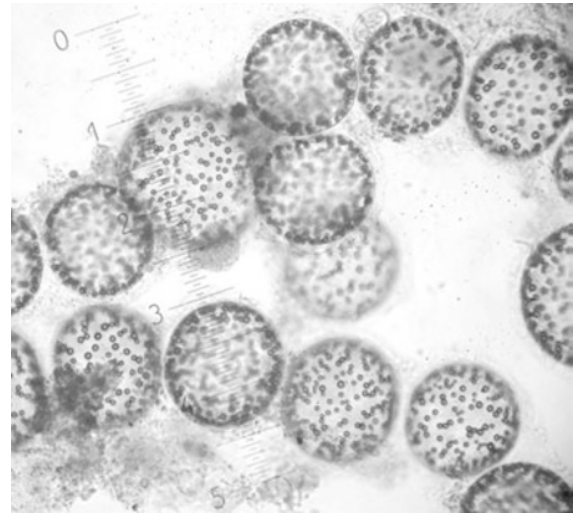


Fig. 11. Light micrograph of a typical ensheathed chain of *Thiomargarita namibiensis* cells (0.1–0.15 mm in diameter). Courtesy of Heide Schulz.

Table 1. Characteristic *Beggiatoa* and *Thioploca* species and strains.

Name	Filament diameter (µm)	Habitat	Cytoplasmic vacuolation	NO <sub>3</sub> <sup>-</sup>	Autotrophy accumulation	References
<i>Thioploca</i>						
<i>T. schmidlei</i>	5–9	Freshwater	ND	Unknown	Unknown	Lauterborn, 1907
“ <i>T. minima</i> ”	1–2	Freshwater	ND	Unknown	Unknown	Maier, 1984
<i>T. ingraca</i>	2–5	Fresh/brackish	Existent	Unknown	Unknown	Maier and Murray, 1965
<i>T. spp.</i>	2–5	Freshwater vent	Existent	136mM	Unknown	Zemskaya et al., 2001
<i>T. marina</i>	2.5–5	Marine	ND	Unknown	Unknown	Schulz et al., 2000
<i>T. chileae</i>	12–20	Marine	Dominates cytoplasm	Max. 500mM	Yes	Maier and Gallardo, 1984 Otte et al., 1999
<i>T. araucae</i>	30–43	Marine	Dominates cytoplasm	Max. 500mM	Yes	Maier and Gallardo, 1984 Otte et al., 1999
<i>Beggiatoa</i>						
Str. MS-81-1c	1.6–2.2	Marine	No	≤0.3µM	Obligate	Nelson and Jannasch, 1983
Str. MS-81-6	4–5	Marine	No	ND	Facultative	Nelson and Jannasch, 1983
Str. D-402	ND	Freshwater	No	ND	Facultative	Patritskaya et al., 2001
Str. OH-75-2a	1.5–2.3	Freshwater	No	ND	Heterotroph	Nelson and Castenholz, 1981b
<i>Beggiatoa alba</i> (type strain B18LD)	3.5	Freshwater	No	ND	Heterotroph	Mezzino et al., 1984
Str. L1401-13	2.5	Freshwater	No	ND	Heterotroph	Pringsheim, 1964
Monterey Canyon	65–85	Marine	Yes	160mM	Yes	McHatton et al., 1996
Guaymas Basin	90–140	Marine	Yes	130mM	Yes	Nelson et al., 1989

Abbreviation: ND, no data.

1984). This is at odds with physiological, genomic and morphological evidence for considerable diversity within the genus *Beggiatoa*. Several important freshwater and marine *Beggiatoa* strains with contrasting physiological properties (from obligately autotrophic to heterotrophic growth) have been investigated and described in detail (Table 1). Also, discrete size classes of *Beggiatoa* filaments have been described early (Klas, 1937), and the identification of ever larger size classes has extended the size spectrum of filament diameters to ca. 200  $\mu\text{m}$  (Nelson et al., 1989c; Larkin and Henk, 1996). For uncultured *Beggiatoa* spp., a consistent taxonomy could be developed on the basis of reproducibly defined morphotypes correlated with 16S rRNA sequences.

For both *Beggiatoa* and *Thioploca*, marine and freshwater habitats harbor different physiological and morphological types (Table 1). In contrast to small freshwater *Thioploca* with filament diameters in the range of 1–9  $\mu\text{m}$ , marine *Thioploca* species reach diameters of at least 20–40  $\mu\text{m}$ . Similarly, marine *Beggiatoa* populations can reach filament diameters of 100–200  $\mu\text{m}$  (Table 1). Large *Thioploca* and *Beggiatoa* spp. have large cytoplasmic vacuoles that comprise ca. 80–90% of the cellular biovolume (Maier et al., 1990; McHatton et al., 1996). So far, very high intracellular nitrate concentrations of ca. 100–500 mM have been found in all environmental populations of large, vacuolated *Beggiatoa* and *Thioploca*, and in a population of freshwater *Thioploca* that match the size class of *Thioploca ingrca* (Zemskaya et al., 2001). These *Thioploca* and *Beggiatoa* species accumulate and store nitrate intracellularly, most likely in their vacuoles. On the basis of these phenotypic and physiological similarities, large, vacuolated, nitrate-accumulating *Thioploca* and *Beggiatoa* populations could be specifically related to each other and to *Thiomargarita*. This grouping is confirmed by 16S rRNA sequence analyses that show a monophyletic branch for these populations. Implicitly, sheath formation, the classical character to separate *Beggiatoa* from *Thioploca*, appears to be phylogenetically shallow and variable (Teske et al., 1995; Teske et al., 1999; Ahmad et al., 1999b). These physiologically and phylogenetically consistent characters may in the future provide a basis to revise the delineation between the genera *Beggiatoa* and *Thioploca*.

## Cell Structure

Narrow *Beggiatoa* filaments examined in pure culture (2–5  $\mu\text{m}$  width) are typically composed of cylindrical cells, with lengths from 1.5–8  $\times$  their width (Faust and Wolfe, 1961; Scotten and

Stokes, 1962; D. Nelson, unpublished observations). However, crosswalls are rarely visible in cultured cells filled with poly- $\beta$ -hydroxybutyrate (PHB) or elemental sulfur (Figs. 6 and 7). Trichomes may exceed 1 cm in length when actively growing in semisolid medium. Terminal cells are rounded in all cultured strains. Exhausting of nutrients results in trichome breakage at necridia or “sacrificial” cells (Pringsheim, 1964; Strohl and Larkin, 1978a) to produce trichomes as short as 3–10  $\mu\text{m}$  in length. The wider filaments of large marine *Beggiatoa* are disk-shaped with cell lengths from 0.10–0.90  $\times$  their cell width (Table 1). Cells of large, marine *Beggiatoa* filaments are hollow, i.e., composed of a thin cylinder of cytoplasm surrounding a large central vacuole. This extensive vacuolation, combined with high intracellular nitrate concentration, is consistently found in large *Beggiatoa* and *Thioploca* (Janasch et al., 1989b; Nelson et al., 1989c; Larkin and Henk, 1996; McHatton et al., 1996).

The few *Beggiatoa* strains tested are Gram negative both phylogenetically and by staining; however, they have unusual cell wall structures. Figure 12 shows a schematic diagram of *Beggiatoa alba* B15LD that indicates that only the inner layer of the cell wall (presumably murein) plus the cell membrane participate in septation. The outer envelope layers do not participate in cross-wall formation but are apparently continuous over the entire filament length (Strohl et al., 1982).

Three types of inclusions have been reported for various *Beggiatoa* strains: PHB (Pringsheim, 1964; Strohl and Larkin, 1978b; Strohl et al., 1982), polyphosphate (Maier and Murray, 1965; Strohl and Larkin, 1978b), and sulfur (Winoogradsky, 1887; Strohl et al., 1981b; Strohl et al., 1982). The reports of polyphosphate should be regarded as tentative because the staining technique employed is nonspecific (Krieg and Hylmon, 1976). Production of PHB appears, on the other hand, to be a universal feature of the heterotrophic freshwater strains examined. Numerous cell inclusions that most likely are PHB were also found by transmission electron microscopy (TEM) in the fresh and brackish water species *Thioploca ingrca* (Kojima et al., 2003). Interestingly, PHB deposition in *Beggiatoa* seems to correlate primarily with high aeration (Pringsheim, 1964), and it can account for up to 50% of total dry weight under these conditions in the absence of sulfide (Güde et al., 1981).

The sulfur inclusions of *Beggiatoa* are periplasmic in location, being enclosed in invaginations of the cell membrane (Fig. 12). The sulfur globules in the specific strain diagrammed here are enclosed within a multilayered sulfur inclusion envelope of 12–14 nm thickness (Strohl et al., 1982), while in other strains the S<sub>0</sub> globule enve-



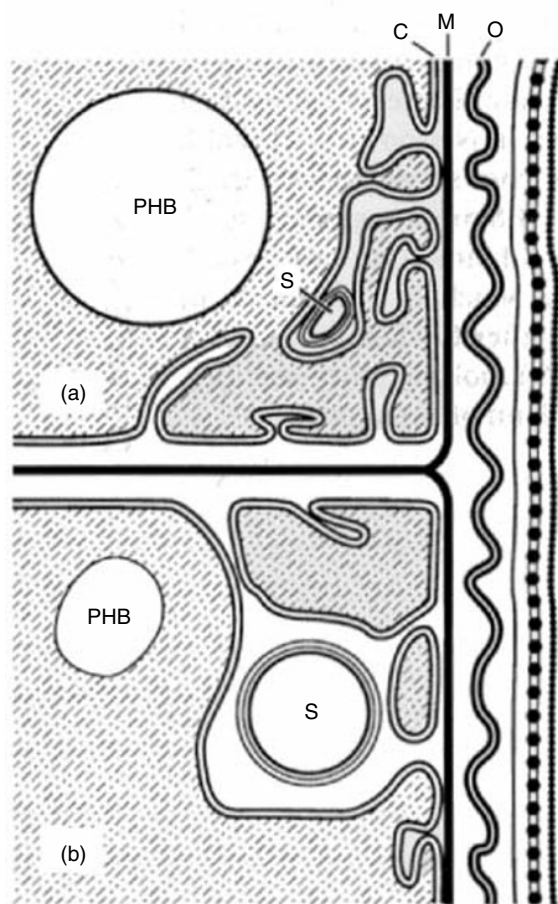


Fig. 12. Ultrastructure of *Beggiatoa alba* strain B15LD (ATCC 33554). Symbols: C, cell membrane; M, presumed murein layer; O, presumed outer membrane layer; S, globule of S<sub>0</sub>; PHB, poly-β-hydroxybutyrate granule. A) Note large PHB inclusion and rudimentary S<sub>0</sub> globule typical of cells grown in acetate-supplemented mineral medium. B) Note small PHB inclusion and large S<sub>0</sub> globule typical of cells grown in the presence of sulfide or thiosulfate and a low concentration of acetate. Adapted from Strohl et al. (1982).

lope appears to be composed of a single protein layer 4–5 nm thick (Strohl et al., 1981b). The extraction of S<sub>0</sub> globules with solvents such as pyridine and their refractile appearance when intact cells are viewed under phase contrast microscopy have proven very useful in confirming their presence (Skerman et al., 1957). As shown schematically (Fig. 12), *Beggiatoa* cells grown in the absence of reduced sulfur compounds apparently contained small, rudimentary S<sub>0</sub> inclusion envelopes (Strohl et al., 1982). Since dehydration solvents (e.g., ethanol) necessary for preparation of electron microscopy dissolve the S<sub>0</sub>, it is difficult to determine whether the rudimentary inclusions completely lack elemental sulfur. The sulfur globules consist of fine grained, microcrystalline elemental sulfur in the common,

stable S<sub>8</sub> ring configuration (Pasteris et al., 2001; Prange et al., 2002) without significant additions of other elements (Lawry et al., 1981); they are surrounded by extensions of the cytoplasmic membrane plus an inner sulfur inclusion envelope (Lawry et al., 1981; Strohl et al., 1981b). The cyclooctasulfur globules of *Beggiatoa alba* (strain DMSZ 1416) and of *Thiomargarita namibiensis* differ from sulfur globules produced by other sulfur oxidizers, such as polythionate sulfur globules of *Acidithiobacillus thiooxidans*, and sulfur chain-dominated sulfur globules of anoxygenic phototrophs (Prange et al., 2002); the differences in sulfur speciation probably reflect different sulfur deposition pathways.

At present, no ultrastructural markers are known that separate *Beggiatoa* from *Thioploca* cells and filaments. In other words, a *Thioploca* filament outside its sheath is indistinguishable from a *Beggiatoa* filament. The sheath formed by *Thioploca* spp, while not a structural feature of the cell, is a taxonomically useful marker and structural characteristic that can distinguish *Thioploca* species. The sheaths of *Thioploca ingrica* strains from fresh and brackish water show conspicuous constricted zones at random intervals (Wislouch, 1912; Kojima et al., 2003); this characteristic has not been found in marine *Thioploca* species. *Thioploca* sheaths appear to have a striated texture that runs parallel to the *Thioploca* filaments; epibiotic filamentous bacteria on and within the sheath matrix tend to be aligned parallel to the sheath striation and the *Thioploca* filaments (Fukui et al., 1999). Within a marine *Thioploca* population, the cell dimensions indicate two clearly separated morphotypes, the long-cell and the short-cell morphotype, with ratios of average cell length to trichome diameter above and below 0.48 (Schulz et al., 2000).

## Isolation and Cultivation

### Selective Enrichments

The most consistently successful enrichments for *Beggiatoa* spp. have been made as follows: The bottom of a shallow pan or aquarium (approximately 30 × 30 × 12 cm) is covered with a few centimeters of sand. CaSO<sub>4</sub> (approx. 20 g) and K<sub>2</sub>HPO<sub>4</sub> (a few grams) are then added, along with a source of complex organic polymers such as seaweed or shredded paper. This is covered with several centimeters of sulfide-rich marine mud, and then enough seawater to cover the entire enrichment to a depth of 1–2 cm is added. Covering the pan with aluminum foil or incubating in a dark place minimizes competition with phototrophic bacteria. The enrichment is certain

to contain the proper sulfide-oxygen interface somewhere in the vessel if air is introduced near the sediment surface using an air stone. Water lost by evaporation should be replaced by distilled water. Alternatively, a slow steady flow of freshly aerated seawater, with a drain maintaining a constant level, will provide the necessary  $O_2$ . A similar freshwater enrichment inoculated with mud from a sulfur spring and maintained on a light-dark cycle (10h:14h) provided viable tufts of *Beggiatoa* spp. for almost one a year (Nelson and Castenholz, 1982a). Sewage treatment plants are also an excellent source of enrichment material (Burton and Lee, 1978; Williams and Unz, 1985).

Another type of enrichment has a long historical association with *Beggiatoa* and is based on the use of extensively extracted dried grasses or hay in an otherwise mineral medium (Cataldi, 1940). The complex polymers such as cellulose residues in the material presumably fuel sulfate reduction localized near the surface of the grasses. This, in turn, provides the hydrogen sulfide necessary to enrich for *Beggiatoa*. The grasses also serve as a physical substrate for these gliding bacteria. Inclusion of soil extract and the enzyme catalase appear to enhance the rate and success of this enrichment (Joshi and Hollis, 1976; Strohl and Larkin, 1978b).

Without attempts at enrichments or pure culture isolation, *Beggiatoa* mats growing on sulfidic sediments kept alive for some time in an aquarium in their natural sediment. The development of a marine *Beggiatoa* mat in the laboratory unfolded over 10 days and revealed a succession of different *Beggiatoa* size classes, with larger types replacing the smaller ones (Bernard and Fenchel, 1995).

### Pure Culture Isolation

Three different approaches have been used to isolate and to maintain *Beggiatoa* strains in pure culture.

**ISOLATIONS ON AGAR PLATES** Tufts of *Beggiatoa* filaments are collected from the environment or an enrichment with a Pasteur pipette or pointed forceps, washed by multiple rinses in sterile washing solution, and placed on the center of a well-drained agar plate (0.8–1.2% agar) that contain dilute organic substrates, such as small amounts (1–0.25% w/v) of peptone or yeast extract. Growing filaments that move away from the central inoculum are cut out on agar blocks and are used as inoculum for new agar plates (Pringsheim, 1967). These procedures have led to the isolation of heterotrophic *Beggiatoa* strains. Modifications of this technique were aimed at reducing the organic carbon content of

the agar medium by using defined mineral media (Nelson and Castenholz, 1981a) to select against heterotrophic contaminants, and to favor enrichment and isolation of autotrophic *Beggiatoa*. Agar plates (made with filtered seawater, trace elements, vitamin mix, and supplemented with sodium sulfide, ammonium sulfate, sodium thiosulfate, and sodium acetate) were used for the isolation of marine *Beggiatoa* strains that in subsequent tests showed autotrophic growth (Nelson et al., 1982b). While the isolation of freshwater strains has typically employed agar-gelled medium equilibrated with full air, the use of bell jars to provide a micro-oxic atmosphere has, thus far, been essential for isolation of autotrophic marine strains.

For the freshwater strains currently available, isolation was performed under oxic conditions (air atmosphere) on a variety of media that contained a low concentration of a single organic compound, principally acetate, lactate or glucose, and sometimes a reduced sulfur source, such as  $Na_2S$  or thiosulfate (Strohl and Larkin, 1978b; Nelson and Castenholz, 1981a; Williams and Unz, 1985). A representative medium is shown here:

#### DTA Medium

ND stock solution	50 ml
$(NH_4)_2SO_4$	0.13 g
Sodium acetate	0.68 g
$K_2HPO_4$	0.027 g
$Na_2S_2O_3 \cdot 5H_2O$	0.50 g
$CaCl_2$	0.10 g
Distilled water	950 ml
Agar	8–12 g

#### ND Stock Solution (Castenholz, 1988)

Distilled water	1000 ml
NTA (nitrilotriacetic acid)	2.0 g
Micronutrient solution	10 ml
$FeCl_3$ solution (0.29 g/liter)H	20 ml
$CaSO_4 \cdot 2H_2O$	1.2 g
$MgSO_4 \cdot 7H_2O$	2.0 g
NaCl	0.16 g
$Na_2HPO_4$	1.4 g
$KH_2PO_4$	0.72 g

#### Micronutrient Solution

Distilled water	1000 ml
$H_2SO_4$ (concentrated)	0.5 ml
$MnSO_4 \cdot H_2O$	2.28 g
$ZnSO_4 \cdot 7H_2O$	0.50 g
$H_3BO_3$	0.50 g
$CuSO_4 \cdot 5H_2O$	0.025 g
$Na_2MoO_4 \cdot 2H_2O$	0.025 g
$CoCl_2 \cdot 6H_2O$	0.045 g

The pH is adjusted to 7.0 prior to autoclaving. As in the marine isolations, it is important that the surface of the agar is dry. For purification of single filaments from enrichment-derived tufts, lowering the acetate concentration to 0.5 mM for the initial dispersal of filaments may minimize contamination.

A sulfide-oxygen gradient medium based on DTA medium has proved effective for maintaining freshwater strains because they require infrequent transfer. The sulfide concentration of the butt should be reduced to 3–4 mM, and thiosulfate can be omitted from the top agar.

**ISOLATION OF LITHOTROPHIC MARINE STRAINS** The known marine strains of *Beggiatoa* are obligate microaerophiles that were isolated under micro-oxic conditions (4 kPa O<sub>2</sub>) on medium supplemented with sodium sulfide and sodium acetate. Subsequently, it was realized that all strains will grow luxuriantly in sulfide-gradient medium (described below) in the absence of added organics.

The following procedure ensures a marine basal medium (J3) free of precipitates.

#### J3 Medium

##### Solution 1

Prefilter (Whatman #1 or Gelman GF/F) then filter (0.45 μm) 500 ml of aged natural seawater (salinity 3.2–3.5%).

##### Solution 2

Dissolve 9.0 g of agar in 200 ml of distilled water.

##### Solution 3

Mix the following ingredients: NH<sub>4</sub>NO<sub>3</sub>, 0.06 g; trace elements (SL8; Pfennig and Biebl, 1981), 0.75 ml; and mineral stock, 50 ml. The mineral stock contains per liter: K<sub>2</sub>HPO<sub>4</sub>, 0.52 g; Na<sub>2</sub>MoO<sub>4</sub>, 0.05 g; FeCl<sub>3</sub> · 6H<sub>2</sub>O, 0.29 g; Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (sodium pyrosulfite), 0.75 g; and phenol red, 10 ml of a sterile solution (0.5%, Gibco).

Autoclave solutions 1, 2 and 3 separately in Erlenmeyer flasks. After cooling to 50°C, aseptically combine in the solution 2 vessel (volume >750 ml). Then supplement with 0.2 ml of Va vitamin solution, which contains (in mg per liter): B12, 1; thiamine, 200; biotin, 1; folic acid, 1; *para*-aminobenzoic acid, 10; nicotinic acid, 100; inositol, 1; and calcium pantothenate, 100.

#### J-TS Medium

J3 basal medium is amended to produce an isolation medium (J-TS) by adding the following sterile stocks, with final concentrations in parentheses:

##### Solution 1

7.5 ml of 200 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (2 mM)

##### Solution 2

3.75 ml of freshly neutralized 200 mM Na<sub>2</sub>S (1 mM).

Autoclave as a basic solution, which is quite stable against autooxidation, and then neutralize with an equimolar quantity of sterile HCl just prior to use.

##### Solution 3

15 ml of 1 M NaHCO<sub>3</sub> (20 mM). To make this stock, autoclave 8.4 g of NaHCO<sub>3</sub> (dry) and add 100 ml sterile water when cool.

Immediately after solidification, incubate plates in a bell jar for 24 h or more under anoxic conditions (99.5% N<sub>2</sub>, 0.5% CO<sub>2</sub>), with desiccant present to absorb water evaporating from the surface of the medium. The medium is buffered by the bicarbonate in conjunction with the level of atmospheric CO<sub>2</sub>. After inoculation with a tuft of *Beggiatoa* spp., place plates in a micro-oxic atmosphere (0.5% CO<sub>2</sub>; 0.2% O<sub>2</sub>; and 99.3% N<sub>2</sub>). Exposing the

medium and bacteria to full air for approximately 20 min every day or two, as needed for inoculation or single-filament isolations, poses no problem to the success of the technique. Pure cultures resulting from repeated single-filament isolations can be maintained in sulfide-oxygen gradient media (see ISOLATION USING LIQUID MEDIA).

**ISOLATION USING LIQUID MEDIA** Liquid media can be used for enrichment, MPN enumeration, and bulk cultivation of *Beggiatoa*. In an extensive study, Strohl and Larkin (1978b) have tested several liquid media formulations for isolation and MPN enumeration of heterotrophic *Beggiatoa* filaments from organic-rich freshwater ditches and lakes. They obtained the best results with a soil extract amended with 0.05% (w/v) acetate, 15–35 U of catalase per ml, and 1% (w/v) hay extract. Early attempts to use liquid media for bulk cultivation (Kowallik and Pringsheim, 1966) had already demonstrated the importance of small amounts of carbon substrates, either soil or hay extracts or small amounts of acetate, for successful cultivation of heterotrophic or mixotrophic freshwater *Beggiatoa*. The type species and strain (*Beggiatoa alba* str. B18LD) and related strains are generally grown in media that include a salt base, acetate as carbon source, and variable yeast extract and sulfide additions (Mezzino et al., 1984; Schmidt et al., 1986). Only recently, marine autotrophic *Beggiatoa* strains were cultured in bulk in a defined liquid mineral medium with thiosulfate as sole energy source and CO<sub>2</sub> as sole carbon source, under micro-oxic conditions under aeration with 0.25% O<sub>2</sub> (v/v) in the gas phase (Hagen and Nelson, 1996). The cultivation of a freshwater *Beggiatoa* strain in liquid mineral medium on thiosulfate and HCO<sub>3</sub><sup>-</sup> required dissolved oxygen concentrations in the range of 3–16 μM (0.1–0.5 mg O<sub>2</sub>/liter; Patrinskaya et al., 2001).

**ISOLATION AND CULTIVATION IN GRADIENT MEDIA** After single-filament isolations on solid agar medium yielded pure cultures of nonvacuolate *Beggiatoa* spp., autotrophic strains were most easily maintained and propagated in sulfide gradient tubes (Nelson and Jannasch, 1983), where sulfide-rich agar plugs are overlaid with sulfide-free soft agar. For cultivation of estuarine strains, the soft agar medium is based on a 2/3-strength natural seawater medium that lacks reduced sulfur compounds but includes trace elements and vitamin mix, and ammonium nitrate (J2 medium). Tubes were loosely capped to permit exchange of headspace gasses with the atmosphere. The resulting two layers of agar contain opposed sulfide and oxygen gradients, that allow the growth of a well-defined *Beggiatoa* layer at the sulfide-oxygen

interface (Nelson et al., 1986b; Nelson et al., 1986a). Propagation of vent or seep (fully marine) strains of *Beggiatoa* may require medium of a higher salinity.

**CONSTRUCTION OF GRADIENT MEDIUM** Marine gradient medium (JG8) was constructed as follows: First, 4 ml of J3 medium (pH 8.4; 1.5% agar; and  $\text{NaHCO}_3$  concentration lowered to 2.0 mM) supplemented with freshly neutralized  $\text{Na}_2\text{S}$  was solidified in the bottom of a 16 × 150 mm screw-capped tube. An initial sulfide concentration of 8 mM in this butt has proven satisfactory for all isolates tested. The thiosulfate present in the isolation medium need not be included because the tube geometry provides a sustained flux of sulfide for several weeks. The butt was then overlaid with 8.0 ml of semi-solid J3 medium (0.25% agar;  $\text{NaHCO}_3$  concentration lowered to 2.0 mM; and no sulfide or thiosulfate). At this point the gradient of soluble sulfide (neglecting convective mixing) is theoretically a “step-gradient,” i.e., all of the sulfide will be below the interface between the sulfidic agar plug and the sulfide-free overlay agar. The air headspace reservoir in the top 8 ml of the tube constitutes an oxygen reservoir. Molecular diffusion and nonbiological reaction between sulfide and oxygen gradually alter the gradient shapes as described in detail elsewhere (Nelson et al., 1986b; Nelson et al., 1986a).

Aging new gradient media for 2–3 days prior to inoculation establishes a sulfide-oxygen interface that is quite stable in both position and rates of nutrient flux. The interface is located near the top of the agar column, but the extent of sulfide and oxygen overlap is roughly 6–7 mm in uninoculated medium (Nelson et al., 1986a) as compared with 0.2 mm or less in *Beggiatoa* cultures. Whether inoculated at the surface of this medium or stabbed throughout the upper few centimeters, the filaments rapidly proliferate at the sulfide-oxygen interface, forming a marked layer or “plate,” of 1 mm (maximum thickness). Gliding motility and negative chemotactic responses allow these bacteria to track this interface as it slowly descends, owing to the gradual depletion of the sulfide reservoir.

**CULTIVATION OF THIOPLOCA** Currently, no pure cultures or enrichments of *Thioploca* species or strains exist. All biochemical, physiological and molecular work has been performed on *Thioploca* filaments collected from their natural environment, marine or freshwater sediments. Natural *Thioploca* populations can be kept alive in the laboratory for months or even years. Maier (1989) described the following procedures for cultivation of freshwater *Thioploca*. These may be maintained in jars overlaid with

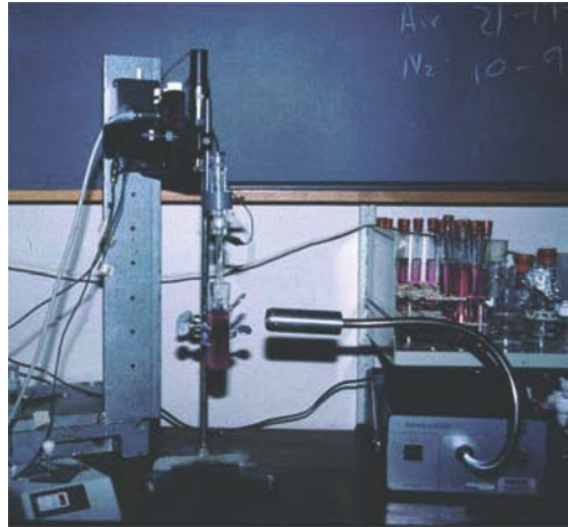


Fig. 13. Gradient culture of marine *Beggiatoa* spp. Photo of Aarhus gradient culture setup. The oxygen profile in a gradient culture of *Beggiatoa* spp. is determined with an oxygen microelectrode mounted onto a micromanipulator (Nelson et al., 1986b). The whitish *Beggiatoa* plate is visible near the surface of the gradient culture, and its position is indicated by the point light source. Courtesy of Douglas Nelson.

tap water at 8–20°C in the dark; at approximately yearly intervals, a few stems of extracted grass (Scotten and Stokes, 1962) may be stuck into the sediment and *Thioploca* often colonizes these stems. Alternatively, 0.2–0.3 g of pulverized extracted hay is autoclaved in 60 ml of tap water in 125-ml Erlenmeyer flasks and inoculated with 4–10 ml of sediment (Maier, 1980). After a month of undisturbed incubation at room temperature to avoid periods of maximum  $\text{H}_2\text{S}$  development, *Thioploca* bundles are added and incubation continues for many weeks with intermittent inspection.

Attempts to enrich marine *Thioploca* spp. have met with little success. They may be maintained for months in undisturbed cores sampled from the natural populations and kept near the in-situ temperature of 13°C in a basin of anoxic seawater with nitrate added (H. Schulz, personal communication). Physiological studies with harvested, large marine *Thioploca* filaments required careful handling of the filaments and avoidance of oxygen and air exposure to avoid significant losses in enzymatic activities (Otte et al., 1999). Future cultivation approaches have to take the sensitivity of *Thioploca* to high sulfide concentrations and to oxygen exposure into account, and maintain the delicate balance of sulfide, nitrate and oxygen concentrations that characterizes its natural habitat (Hüttel et al., 1996; Schulz et al., 2000).

**STRAIN MAINTENANCE** Freshwater and marine strains of *Beggiatoa* spp. are readily maintained in sulfide-oxygen gradient media. The fact that oxygen and sulfide gradients are “self-forming” (Nelson et al., 1986a) coupled with the chemotactic motility of the bacteria, which directs them to the proper micro-environment, makes this approach especially attractive for obligately microaerophilic strains. A low concentration of acetate must be provided for the strains that do not show lithoautotrophic capacity. For the typical medium geometry employed (Nelson, 1992), transfers to new tubes of gradient medium should be made every 2–3 weeks, and medium should be kept no more than 4–6 weeks or the sulfide flux will be greatly diminished. Sustained propagation of heterotrophic freshwater strains on agar plates in the presence of full air is straightforward. By contrast, propagation of marine strains on agar plates under micro-oxic regimes in bell jars is best reserved for initial isolation and any required re-purification steps. Cryopreservation of strains beyond 6 months to one year has proved problematic (D. C. Nelson, unpublished observation).

## Biochemical and Physiological Properties

### Carbon Metabolism

Carbon assimilation processes and pathways of *Beggiatoa* came under investigation shortly after Winogradsky began to develop the concept of microbial chemolithoautotrophy, based on his initial investigations with this organism (Winogradsky, 1887). Almost a century later, the first clearly autotrophic *Beggiatoa* strains were isolated in pure culture (Nelson et al., 1982b; Nelson and Jannasch, 1983). In autotrophic *Beggiatoa* strains, carbon fixation occurs via the Calvin cycle, as judged by the activity level and regulation of RuBPC/O (Ribulose-1,5-bisphosphate carboxylase/oxygenase). In the obligately autotrophic strain MS-81-1c, RuBPC/O cannot be repressed by acetate additions and is always active at similar levels. The facultatively autotrophic strain MS-81-6 tightly regulates autotrophic vs. heterotrophic growth. Acetate additions reduce the activity of RuBPC/O to a small fraction of its activity in organic-free medium, and increase the activity of 2-oxoglutarate dehydrogenase (Hagen and Nelson, 1996), a key enzyme of the citric acid cycle, opens the way to respiratory oxidation of C2 substrates. Under autotrophic growth conditions, 2-oxoglutarate dehydrogenase is not expressed thus interrupting the citric acid cycle at the stage of 2-oxoketoglutarate. As a result, autotrophically

fixed carbon is not oxidized but used for synthesis of cellular compounds. Even the obligately autotrophic *Beggiatoa* str. MS-81-1c increases its growth yield by ca. 20% after addition of acetate, indicating that acetate can be used as an auxiliary carbon source for the synthesis of cell material in a manner analogous to other chemolithoautotrophic sulfur-oxidizing bacteria (Hagen and Nelson, 1996).

Strong evidence for autotrophic potential has been found in uncultured, large marine *Beggiatoa* spp. Freshly collected samples of large, marine *Beggiatoa* from the Guaymas Basin hydrothermal vents showed significant RuBPC/O activity; some of these vent populations possessed RuPBC/O that was active at 50°C and, hence, appear to be moderate thermophiles (Nelson et al., 1989c). Uncultured populations of large *Beggiatoa* from cold sulfide seeps in the Monterey Canyon also showed reasonably high RuBPC/O activity (McHatton et al., 1996; Table 2). The <sup>13</sup>C-isotopic signature of large *Beggiatoa* filaments from cold seep sediments in the Gulf of Mexico was consistent with carbon assimilation by the Calvin cycle (Larkin et al., 1994). *Beggiatoa* mats growing on and within sediment layers with active sulfate-dependent methane oxidation take up <sup>13</sup>C-depleted CO<sub>2</sub> derived from methane oxidation and show the isotopically light signature (δ<sup>13</sup>C in the range of –50 to –60 ‰) of partially methane-derived biomass (Paull et al., 1992; Orphan et al., 2002).

In freshly collected natural samples of marine *Thioploca* spp., CO<sub>2</sub> uptake occurred at rates similar to those of large hydrothermal vent *Beggiatoa* (Otte et al., 1999). Acetate was incorporated into *Thioploca* filaments at a rate roughly equal to that of CO<sub>2</sub>, with no apparent terminal oxidation, suggesting that large marine *Thioploca* spp. are facultative chemolithoautotrophs (Otte et al., 1999). The two dominant Chilean *Thioploca* species (*Thioploca chileae* and *T. araucae*) were both able to incorporate carbonate as well as acetate and amino acids (Maier and Gallardo, 1984), but the conclusions of that study must be tempered by the observation that the inorganic carbon was labeled to a much lower specific activity than the organic substrates (Nelson, 1989a).

**HETEROTROPHIC GROWTH** In contrast to autotrophic marine *Beggiatoa* spp., all but one freshwater *Beggiatoa* strain isolated so far required various organic substrates for growth, at least in small amounts (Faust and Wolfe, 1961; Scotten and Stokes, 1962; Pringsheim, 1964; Burton et al., 1966; Kowalik and Pringsheim, 1966; Strohl and Larkin, 1978; Nelson and Castenholz, 1981a; Nelson and Castenholz, 1981b). Most *Beggiatoa* strains examined can grow with ace-

Table 2. Autotrophic potential in *Beggiatoa* and *Thioploca*.

Name	Electron and carbon sources	RuBPC/O activity (nmol CO <sub>2</sub> fixed min <sup>-1</sup> mg protein)	2-Oxoglutarate dehydrogenase (nmol NAD <sup>+</sup> reduced min <sup>-1</sup> mg protein)
<i>Beggiatoa</i>			
Str. MS-81-1c <sup>a</sup>	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> + acetate	31.7 ± 3.4	0
	H <sub>2</sub> S only	20 ± 2.5	0
	H <sub>2</sub> S + acetate	14 ± 1.6	0
Str. MS-81-6 <sup>a</sup>	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> + acetate	3.4 ± 0.2	103 ± 3.6
	H <sub>2</sub> S only	23 ± 9.2	0
	H <sub>2</sub> S + acetate	4 ± 0.8	153 ± 11.3
Str. OH-75-2a <sup>a,b</sup>	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> + acetate	0.3	100 ± 1.6
	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> + acetate	0.002–0.04 (early growth phase)	n.d.
	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> + acetate	0.2–0.7 (late growth phase)	n.d.
	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> + acetate	0.1–0.2 (early growth phase)	n.d.
<i>Beggiatoa alba</i> <sup>b</sup> strain B18LD	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> + acetate	0.6–1.3 (late growth phase)	n.d.
	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> + acetate	0.6–1.3 (late growth phase)	n.d.
Monterey Canyon <i>Beggiatoa</i> sp. <sup>c</sup> (65–85µm)	in situ	7.5–15	0
Guaymas vents <i>Beggiatoa</i> spp. <sup>d</sup> (25–30µm)	in situ	Approx. 1–2 (at 30°C; 2–4 times more at 50°C)	n.d.
	in situ	1 (at 30°C; none at 50°C)	n.d.
<i>T. araucae</i> and <i>T. chilense</i> <sup>e</sup>	in situ	0.4–0.8	n.d.
<i>Beggiatoa</i>	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> only	73.0 ± 35	n.d.
" <i>leptomitiformis</i> " str. D-402 <sup>f</sup>	S <sub>2</sub> O <sub>3</sub> <sup>-2</sup> + PCMB	5.0 ± 5	n.d.

Abbreviations: RuBPC/O, ribulose-1,5-bisphosphate carboxylase/oxygenase; PCMB, *p*-chloromercuribenzoate (a CO<sub>2</sub> fixation inhibitor); and n.d., no data.

<sup>a</sup>Data from Hagen and Nelson, 1996.

<sup>b</sup>Data from Nelson et al., 1989b.

<sup>c</sup>Data from McHatton et al., 1996.

<sup>d</sup>Data from Nelson et al., 1989a.

<sup>e</sup>Data from Otte et al., 1999.

<sup>f</sup>Data from Patritskaya et al., 2001.

tate as a sole source of carbon and energy. Heterotrophically cultured freshwater *Beggiatoa* strains that were transferred to liquid culture with H<sub>2</sub>S as energy source, grew much better with acetate additions in the range of 0.01–0.0001% (w/v; Kowallik and Pringsheim, 1966). All strains of the type species *Beggiatoa alba* (B18LD, B15LD, and B25RD) grew well in the presence of sulfide and additions of 0.001–0.05% acetate (Mezzino et al., 1984). *Beggiatoa alba* B18LD quickly incorporated <sup>14</sup>C-labelled acetate into a wide range of cellular and storage compounds. The strain had possessed the ability to oxidize acetate (both C-atoms) to CO<sub>2</sub> and to use it as an energy source. Also, acetate significantly increased the capability of this strain to assimilate CO<sub>2</sub>, probably through anaplerotic reactions of the tricarboxylic acid (TCA) cycle (Strohl et al., 1981a).

In the heterotrophic *Beggiatoa* strains that have been tested, carbon substrate utilization patterns are generally consistent with a functional TCA cycle. A detailed study of heterotrophic nutrition of *Beggiatoa* strain OH-75-2a indicated a functional TCA cycle with a

glyoxylate bypass (Nelson and Castenholz, 1981a). *Beggiatoa* str. OH-75-2a can grow on acetate, ethanol, lactate, pyruvate with a small addition of yeast extract, and on TCA cycle intermediates in combination with acetate. A detailed enzymological study with another chemoheterotrophic strain, "*Beggiatoa leptomitiformis*" D-405, confirmed the presence of all essential TCA cycle enzymes during growth on succinate (Grabovich et al., 1993). Furthermore, the presence of enzymes defining the glyoxalate-bypass were detected during growth on acetate or lactate.

Although reliable data are available on heterotrophic and autotrophic capabilities of *Beggiatoa* spp., there is no conclusive evidence for mixotrophic growth of *Beggiatoa*. The term "mixotrophic" is used here in the sense that heterotrophically growing *Beggiatoa* spp. which oxidize organic compounds can to a certain degree substitute sulfide or other reduced sulfur compounds as electron donors. In this way, they can utilize a greater portion of their available (and limited) organic carbon sources for cell material synthesis instead for terminal oxidation. This

type of chemolithoheterotrophy does not require autotrophic CO<sub>2</sub> fixation capabilities, and would be generally possible for heterotrophic *Beggiatoa* strains. The idea is intriguing, since mixotrophic growth should broaden the ecophysiological flexibility of *Beggiatoa* and *Thioploca* spp. in nature (Pringsheim, 1967; Strohl and Schmidt, 1984). So far, quantitative experiments with the heterotrophic strain OH-75-2a have shown that the increased growth yield in dry weight is accounted for by the weight of the accumulated sulfur globules, not by an increase in cellular biomass (Nelson and Castenholz, 1981b). Attempts to demonstrate mixotrophy showed experimental shortcomings and need to be revisited (Güde et al., 1981; see also Nelson and Jannasch, 1983). Sulfur oxidation in heterotrophic strains does not proceed efficiently beyond the stage of elemental sulfur; and the energy-yielding oxidation of sulfur to sulfate is not exploited for mixotrophic growth in the best-studied freshwater strains (Nelson and Castenholz, 1981b; Schmidt et al., 1987). These findings are complemented by a survey of the sulfur-oxidizing enzymes in the heterotrophic freshwater strain OH-75-2a, which does not show activity of sulfite-oxidizing enzymes (no APS reductase and no ADP sulfurylase) that enable the oxidation of sulfite to sulfate (Hagen and Nelson, 1997). However, this strain shows activity of AMP-independent, membrane-associated sulfite:acceptor oxidoreductase, one of the enzymes required for generating energy from the oxidation of reduced sulfur compounds. The facultatively autotrophic marine *Beggiatoa* strain MS-81-6 is most likely mixotrophic with regard to both carbon and energy metabolism (Hagen and Nelson, 1996).

Nothing is known about heterotrophic growth requirements and substrates for *Thioploca*. One may speculate that the smaller, freshwater *Thioploca* species correspond in their physiology to small freshwater heterotrophic *Beggiatoa*; research in this area is lacking so far.

**NITROGEN METABOLISM** *Beggiatoa* and *Thioploca* spp. show considerable versatility in utilizing nitrogen compounds, either as nitrogen source for growth or, in the case of nitrate, as electron acceptor for respiration. All in all, several kinds of nitrogen utilization can be distinguished.

Heterotrophic freshwater *Beggiatoa* spp. assimilate various nitrogen components for growth. *Beggiatoa alba* strains can use nitrate, nitrite, ammonia and casamino acids as sole nitrogen source (Mezzino et al., 1984), and for strain B18LD, the list also includes urea, aspartate, asparagine, alanine and thiourea (Vargas and Strohl, 1985a). *Beggiatoa alba* B18LD assim-

ilates ammonia by the glutamine synthetase–glutamate synthase pathway (Vargas and Strohl, 1985a). Nitrate could not be used as electron acceptor for growth with sulfide oxidation; it allowed a limited degree of acetate oxidation but was not sufficient to sustain growth as the sole electron acceptor. The enzyme activity was associated with the soluble fraction, not with the cell membranes, and produced ammonia as the waste product. On the basis of its cellular localization and biochemical properties, the nitrate reductase of *Beggiatoa alba* appeared to be an assimilatory nitrate reductase (Vargas and Strohl, 1985b).

Nitrogenase activity in *Beggiatoa alba* was strongly regulated by nitrogen compounds. Nitrate and nitrite additions to the growth medium prevented induction of nitrogenase. In vivo nitrogenase activity was only inhibited by ammonia and urea (Polman and Larkin, 1988). Similarly, for the heterotrophic freshwater strain OH-75-2a and for several other strains isolated from a warm freshwater spring (Nelson and Castenholz, 1981a; Nelson and Castenholz, 1981b), and also for the autotrophic marine strains MS-81-1c and MS-81-6, nitrogenase was repressed by nitrate and ammonia in the growth medium (Nelson et al., 1982b). Thus, *Beggiatoa* spp. can contribute to total nitrogen fixation in their natural habitats, including cyanobacterial mats, intertidal sulfureta, and sediments.

The large *Beggiatoa* and *Thioploca* species that accumulate nitrate intracellularly use it most likely as a respiratory electron acceptor for sulfur oxidation (Fig. 14). The large marine *Beggiatoa* sp. from Monterey Canyon, with an intracellular nitrate content of ca. 160 mM, showed the highest level of nitrate reductase in comparison to other strains. Nitrate reductase activity was predominantly found in the particulate fraction, indicating a membrane-bound location within the respiratory chain (McHatton et al., 1996).

A respiratory role for nitrate is also strongly supported for large *Thioploca* spp. When freshly collected filaments of large marine *Thioploca* spp. were incubated with 15N-labeled nitrate, the *Thioploca* cells accumulated labeled nitrate in their vacuoles and reduced it mainly to ammonia. A small amount of dinitrogen was produced, but in this contaminated natural assemblage it cannot be assigned with assurance to the metabolism of *Thioploca* (Otte et al., 1999). Owing to nitrate ammonification by *Thioploca*, ammonia accumulates at high rates in *Thioploca*-harboring sediment surface layers (Thamdrup and Canfield, 1996). The ammonia produced can be readily reassimilated by other bacteria or reoxidized to nitrate by other chemoautotrophs at the sediment water interface. Thus, nitrogen com-

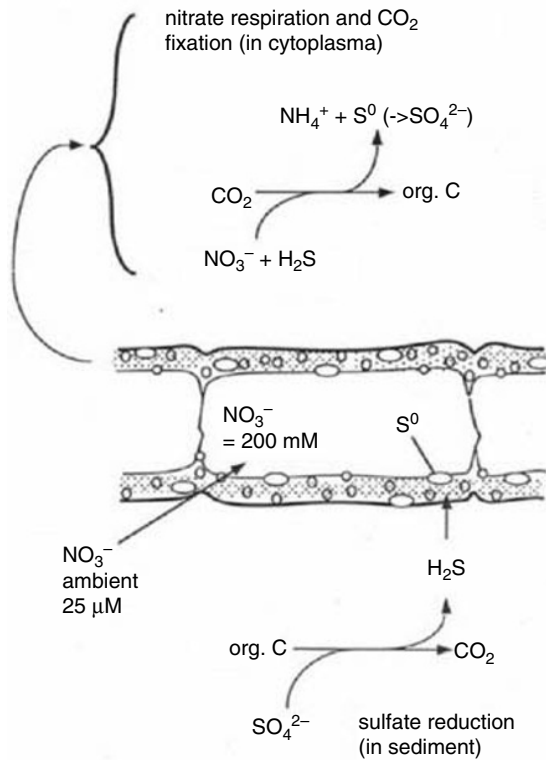


Fig. 14. Links between nitrogen, carbon and sulfur metabolism in large, marine *Beggiatoa*, *Thioploca* and *Thiomargarita* spp.

pounds are not lost from the ecosystem via denitrification (Farias et al., 1996; Farias, 1998).

**SULFUR METABOLISM** The formation of intracellular sulfur globules by oxidation of reduced sulfur sources is one of the basic, defining features of the genera *Beggiatoa* and *Thioploca*. The physiological roles of sulfide oxidation and sulfur accumulation are complex and diverge between different physiological types of *Beggiatoa* spp (Fig. 15). In autotrophic *Beggiatoa*, sulfide is a source of energy and electrons for carbon fixation and growth. Autotrophic sulfur oxidation was first studied in *Beggiatoa* str. MS-81-6 growing in sulfide gradient cultures, where *Beggiatoa* filaments grew as a defined band in a slush soft agar column at the sulfide-oxygen interface (Nelson and Jannasch, 1983). The *Beggiatoa* filaments in the gradient culture migrated over time and their position remained at the sulfide/oxygen interface. Their growth depended on availability of both compounds, oxygen and sulfide, in opposed overlapping gradients (Nelson and Jannasch, 1983; Nelson et al., 1986a). *Beggiatoa* str. MS-81-6 showed considerable flexibility in adjusting the oxidation pathways of sulfide during different

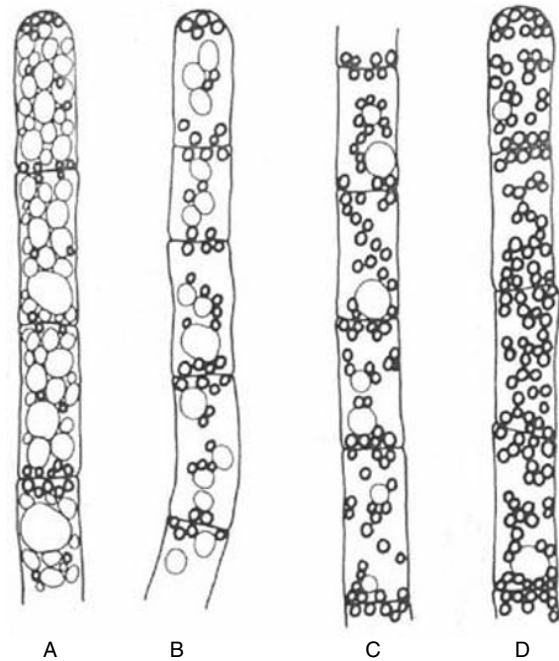


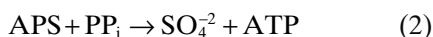
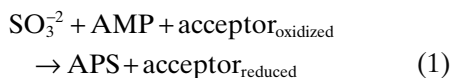
Fig. 15. *Beggiatoa* trichomes f strain 1401-13 grown under different acetate concentrations (w/v) in the presence of H<sub>2</sub>S in liquid medium. A) 0.01%; B) 0.001%; C) 0.0001%; and D) no acetate. Sulfur granules are drawn in bold outlines, and poly-β-hydroxybutyrate bodies in thin outline. Drawing from Kowallik and Pringsheim (1966).

growth stages. Sulfide oxidation could lead to the stage of elemental sulfur, which maximized hydrogen sulfide removal when this compound was overabundant. Alternatively, sulfide could be completely oxidized to sulfate, which used sulfide most efficiently as electron donor when the supply was limited (Nelson et al., 1986a). Sulfide is biologically oxidized at a rate that is roughly three orders of magnitude faster than the competing chemical oxidation, with half-life times of a few seconds in the oxygen-sulfide transition zone (Nelson et al., 1986a). In comparison to other autotrophic sulfur-oxidizing bacteria, both marine autotrophic *Beggiatoa* strains tested have high molar growth yields (8 g/mol for *Beggiatoa* str. MS-81-6, and 16 g/mol for MS-81-1c) on sulfide in gradient cultures (Nelson et al., 1986a; Hagen and Nelson, 1997).

Physiological differences between the obligately autotrophic strain (MS-81-1c) and the facultatively autotrophic strain (MS-81-6) are apparent in different enzyme systems for sulfur oxidation (Hagen and Nelson, 1997). *Beggiatoa* strain MS-81-1c uses APS reductase (adenosine 5'-phosphosulfate reductase; located in the cytosol) in the AMP-dependent oxidation of sulfite to APS. In a second step catalyzed by the enzyme



ATP sulfurylase, the pyrophosphate-dependent substrate-level phosphorylation of APS produces ATP and sulfate:



Both enzymes are highly active regardless of the sulfur source ( $\text{H}_2\text{S}$  gradient, thiosulfate, or thiosulfate with acetate). Substrate-level phosphorylation during sulfur oxidation opens a new source of energy for this *Beggiatoa* strain, in contrast to other *Beggiatoa* strains that appear to lack this pathway and depend on respiratory sulfur oxidation. *Beggiatoa* strain MS-81-6 completely lacks APS reductase activity. The activity of the ATP sulfurylase is two orders of magnitude lower than in strain MS-81-1c, and in the typical range for assimilatory ATP sulfurylases. An assimilatory role for the ATP sulfurylase is supported by the ability of strain MS-81-6 to grow with acetate on sulfate as the only sulfur source (Nelson and Jannasch, 1983).

An AMP-independent, apparently membrane-associated, sulfite:acceptor oxidoreductase system represents a second sulfur oxidation pathway, which is found in *Beggiatoa* strains MS-81-1c, MS-81-6, and OH-75-2a. Since these sulfite oxidases are localized in the cell membrane, it is very likely that they are integrated with the respiratory chain and use cytochrome *c* as electron acceptor. In strain MS-81-1c, sulfite:acceptor oxidoreductase is upregulated in the presence of  $\text{H}_2\text{S}$  and is at least three times higher than in strain MS-81-6. The different rates of respiratory sulfur oxidation, and additional substrate phosphorylation coupled to sulfite oxidation by the APS reductase system in strain MS-81-1c, probably contribute to the differences in molar growth yield between strains MS-81-1c and MS-81-6 (Hagen and Nelson, 1997).

Interestingly, the heterotrophic freshwater *Beggiatoa* strain OH-75-2a showed a similar spectrum of sulfur-oxidizing enzymes as the facultative autotroph. AMP-independent sulfite:acceptor oxidoreductase was present and active in a similar range as in strain MS-81-6, allowing in principle the energy-gaining oxidation of sulfur compounds. However, the activities of ATP sulfurylase were an order of magnitude lower than in strain MS-81-6, and three orders of magnitude lower than the ATP sulfurylase in strain MS-81-1c; thus, the ATP sulfurylase appears to be assimilatory rather than dissimilatory (Hagen and Nelson, 1997).

The sulfur-oxidizing enzyme system of the facultatively autotrophic freshwater *Beggiatoa* strain D-402 shared important features with strains MS-81-6 and OH-75-2a. AMP-dependent

APS reductase was absent; sulfur-oxidizing enzymes that are not involved in substrate-level phosphorylation (sulfite:cytochrome *c* oxidoreductase and thiosulfate:ferricyanide oxidoreductase) were active and were upregulated under autotrophic cultivation conditions with thiosulfate as sulfur source (Grabovich et al., 1998; Grabovich et al., 2001; Patriitskaya et al., 2001). The unusually high activities of RuBPC/O and of sulfur-oxidizing enzymes in strain D-402 could be connected to its high growth yield (12.2 g/mol of oxidized thiosulfate). Direct comparisons of *Beggiatoa* strain D-402, MS-81-1c, MS-81-6 and OH-75-2a with identical culture conditions, sulfur sources, and enzyme assays are necessary to determine the physiological diversity and activity of sulfur-oxidizing enzymes in marine and freshwater *Beggiatoa*.

Sulfur metabolism in other heterotrophic *Beggiatoa* strains differs considerably from sulfur oxidation in autotrophic strains. The heterotrophic *Beggiatoa* strain OH-75-2a was quantitatively studied for sulfide and thiosulfate oxidation effects. Here, sulfide oxidation did not result in additional biomass yield beyond that obtained from oxidation of organic carbon sources; in other words, a mixotrophic growth enhancement by sulfide oxidation that was often proposed by nonquantitative studies of *Beggiatoa* spp. was not found (Nelson and Castenholz, 1981b). The oxidation of sulfur compounds had other functions in this *Beggiatoa* strain. Thiosulfate additions and catalase additions reduced the lag time in the growth of new *Beggiatoa* cultures, suggesting that thiosulfate acts like catalase as a detoxifying agent and electron acceptor for the oxidation of peroxides. Sulfur granules could also serve as an electron acceptor reserve that allows a rudimentary anaerobic respiration with sulfur. *Beggiatoa* str. OH-75-2a used sulfur globules that were accumulated during aerobic thiosulfate oxidation, to sustain anaerobic metabolism and growth during several days of anoxia (Nelson and Castenholz, 1981b). Reduction of sulfur globules to sulfide, coupled to de novo synthesis of cell material, was also found in *Beggiatoa alba* B18LD during anoxic incubation (Schmidt et al., 1987). This mechanism could occur in numerous, so far untested strains of heterotrophic, freshwater *Beggiatoa* spp., and would help the filaments to survive periods of anoxia in their natural interface habitat (Schmidt et al., 1987). However, *Beggiatoa alba* B18LD remains dependent on oxygen for sulfide (and acetate) oxidation. As in autotrophic *Beggiatoa* spp., sulfide oxidation required oxygen as electron acceptor. *Beggiatoa alba* B18LD harbors *c*-type cytochromes that could play a role in respiratory sulfur oxidation (Cannon et al., 1979). There is circumstantial evidence that acetate and sulfide

oxidation compete for oxygen; the addition of acetate and other carbon sources inhibited sulfide oxidation and accumulation of intracellular sulfur globules in *Beggiatoa alba* B18LD considerably (Schmidt et al., 1987). The oxidation of sulfur compounds in *Beggiatoa alba* B18LD stopped essentially at the stage of the elemental sulfur globules. Under a wide range of test conditions, *Beggiatoa alba* B18LD filaments harboring sulfur globules did not release significant amounts of soluble sulfur oxidation products into the surrounding medium (Schmidt et al., 1987).

Oxidation of reduced sulfur compounds is linked to nitrate reduction in large, marine *Beggiatoa* and *Thioploca* spp. Sulfide oxidation and ammonia production rates in marine *Thioploca* samples showed a stoichiometric ratio of ca. 2, suggesting that nitrate reduction to ammonia was accompanied by concomitant sulfide oxidation to sulfur (stoichiometric ratio 1 : 4) and sulfate (ratio 1 : 1); no sulfur intermediates were detected. Apparently, sulfide is oxidized first to elemental sulfur, which acts as an intracellular electron donor reservoir, and then in a second step, to sulfate. In the absence of sulfide, the ratio of ammonia production (requiring 8 electrons per ammonia) and sulfate production (yielding 6 electrons per sulfate) is close to the predicted stoichiometric ratio of 1.3 : 1 (Otte et al., 1999). A similar sulfur oxidation pathway could exist in large, marine *Beggiatoa* spp. which resemble large marine *Thioploca* spp. in vacuolation and nitrate storage capacity (McHatton et al., 1996). In large marine *Beggiatoa* spp. from the Guaymas Basin (30  $\mu\text{m}$  filament diameter, sample 1615), diverse *c*-type cytochromes were found whose hemes have appropriate oxidation-reduction midpoint potentials for respiratory sulfide oxidation (Prince et al., 1988).

## Phylogeny and Taxonomy

As a morphologically conspicuous genus, *Beggiatoa* was at an early stage included in comparative 5S rRNA analysis (Stahl et al., 1987) and identified as a member of the  $\gamma$ -Proteobacteria. By 16S rRNA sequence analysis, *Beggiatoa* and *Thioploca* spp. form a deep branch within the  $\gamma$ -Proteobacteria, a subdivision of the Proteobacteria that harbors numerous free-living and symbiotic types of sulfur-oxidizing bacteria. The filamentous sulfur oxidizer *Thiothrix* is a member of the  $\gamma$ -proteobacterial subdivision, but not related to *Beggiatoa* or *Thioploca* (Teske et al., 1995; Howarth et al., 1999). The large, vacuolated, marine *Beggiatoa* and *Thioploca* species form a well-defined branch that is separated from the smaller, autotrophic and heterotrophic

*Beggiatoa* spp. (Teske et al., 1995; Teske et al., 1999; Ahmad, 1999a; Ahmad et al., 1999b; Mussmann et al., 2003). This branch also includes the genus and species *Thiomargarita namibiensis*, a nonmotile vacuolated sulfide-oxidizing bacterium that relies on its intracellular nitrate storage capacity for survival; *Thiomargarita* holds the current record among prokaryotes for cell size by volume (Schulz et al., 1999). On the basis of 16S rRNA data, considerable phylogenetic depth separates the physiologically divergent, cultured members of the genus *Beggiatoa*, the obligately or facultatively autotrophic marine strains (strains MS-81-1c and MS-81-6), the heterotrophic freshwater *Beggiatoa* strains (str. OH-75-2a, *Beggiatoa alba* B18LD (T), B15LD, and *Beggiatoa* str. 1401-13), and the mutually related, large marine *Beggiatoa* and *Thioploca* strains (Fig. 16). As discussed in the Identification section, the 16S rRNA data will be important for a possible future restructuring of the genera *Beggiatoa* and *Thioploca*.

In parallel to 16S rRNA sequencing, 16S rRNA probes have been designed for fluorescence in situ hybridization (FISH) and discrimination of different *Beggiatoa* and *Thioploca* species and strains (Teske et al., 1995; Teske et al., 1999; Ahmad et al., 1999b; Kojima et al., 2003; Mussmann et al., 2003). Using FISH probes, community level associations and interactions of *Beggiatoa*, *Thioploca*, and sulfate-reducing bacteria could be visualized in detail (Fukui et al., 1999).

Besides 16S rRNA sequencing, little work has been done on the molecular characterization of *Beggiatoa* and *Thioploca*. The molecular mass of the *Beggiatoa alba* genome has been determined by CoT analysis as  $2.02 \times 10^9$ , which corresponds to  $3.03 \times 10^6$  base pairs, similar to *E. coli* (Genthner et al., 1985). The G+C content for the *Beggiatoa alba* strains B18LD, B15LD and B25RD was determined to be 40–42.7 mol%. The phenotypically similar strain L1401-15 had a different G+C content of 51.7 mol% and appeared to be genetically distinct (Mezzino et al., 1984). The three former *Beggiatoa alba* strains contained plasmids with molecular masses of 12.3–12.8  $\times 10^6$  (or 18.9–19.7 kb) with no described function (Minges et al., 1983). Independent analyses (D. C. Nelson, unpublished observation) determined the following mol% G+C values: B18LD (37.1%), B25RD (35.5%), and OH-75-2a (38.5%). *Beggiatoa alba* was included in a polymerase chain reaction (PCR) survey for dissimilatory sulfite reductase, one of the key enzymes of prokaryotic dissimilatory sulfite and sulfate reduction, and reacted negatively (Wagner et al., 1998).

DNA hybridizations determined the presence of dissimilatory ATP sulfurylase genes in dif-

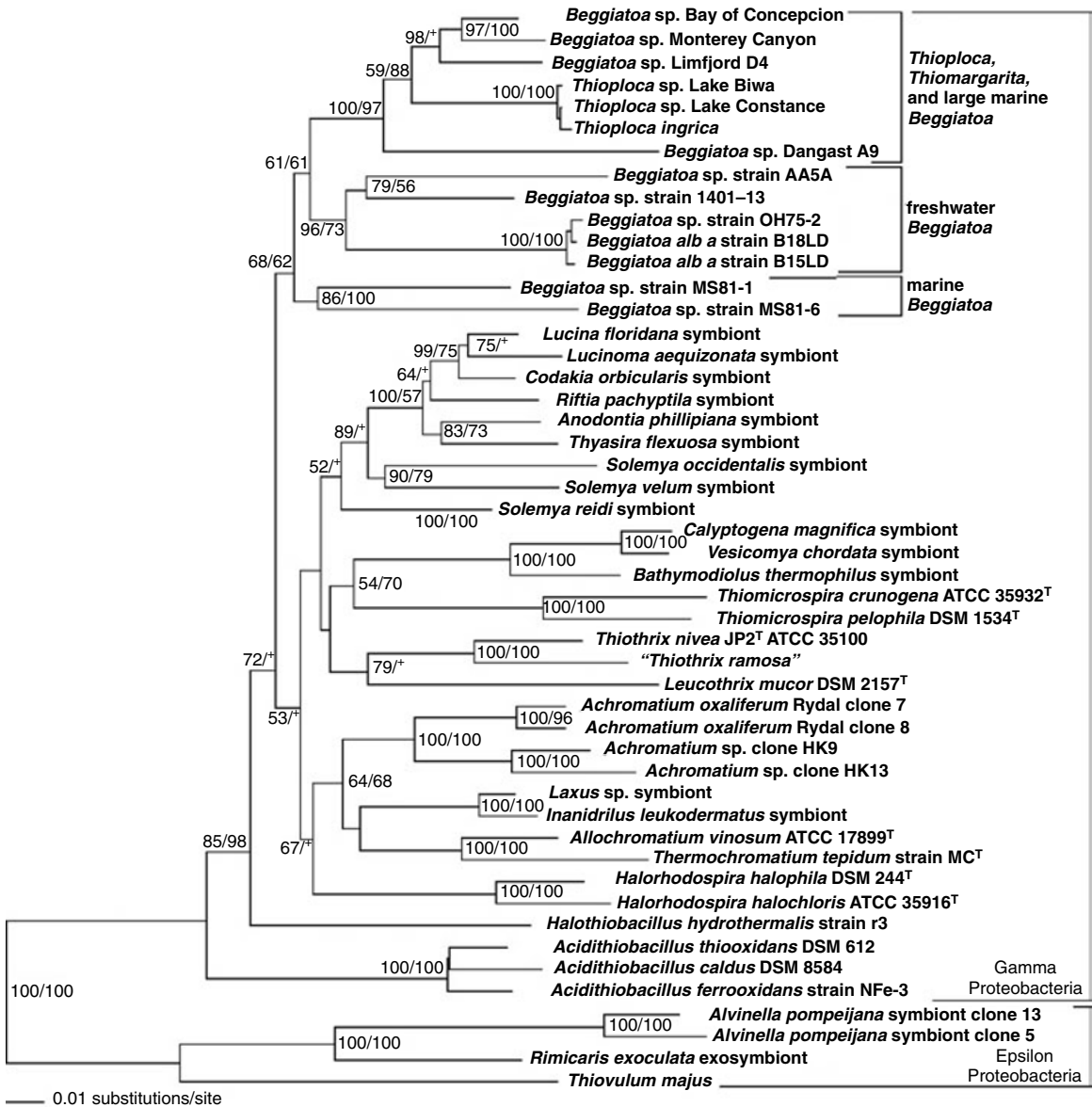


Fig. 16. Phylogeny of the genera *Beggiatoa*, *Thioploca* and *Thiomargarita*, based on A) nearly complete 16S rDNA sequences (*E. coli* positions 28–1487) and B) on partial sequences (*E. coli* positions 341–941), including partial sequences of *Thioploca chileae*, *T. araucae* and *Thiomargarita namibiensis*. Bootstrap values of 1000 replicates (minimum evolution/parsimony) are indicated at nodes with more than 50% bootstrap support.

ferent *Beggiatoa* strains. The gene probe was a fragment of the ATP sulfurylase gene of the autotrophic, sulfur-oxidizing endosymbiont of the hydrothermal vent tube worm *Riftia pachyptila*; the endosymbiont utilizes ATP sulfurylase and APS reductase in dissimilatory sulfur metabolism. DNA of the autotrophic strain MS-81-1c hybridized positively, whereas DNA of the facultatively heterotrophic strain MS-81-6 and of the heterotrophic strain OH-75-2a hybridized negatively, indicating that the latter two strains harbor assimilatory, not dissimilatory ATP sulfurylases (Laue and Nelson, 1994). In general, the

paucity of genetic studies leaves a wide-open field of highly rewarding research, for example a genetic analysis of enzymatic pathways and regulation of the environmentally significant sulfur oxidation pathways.

### Ecology of *Beggiatoa*

*Beggiatoa* and *Thioploca* mats employ different ecophysiological strategies to oxidize sulfide efficiently and to compete with the chemical oxida-

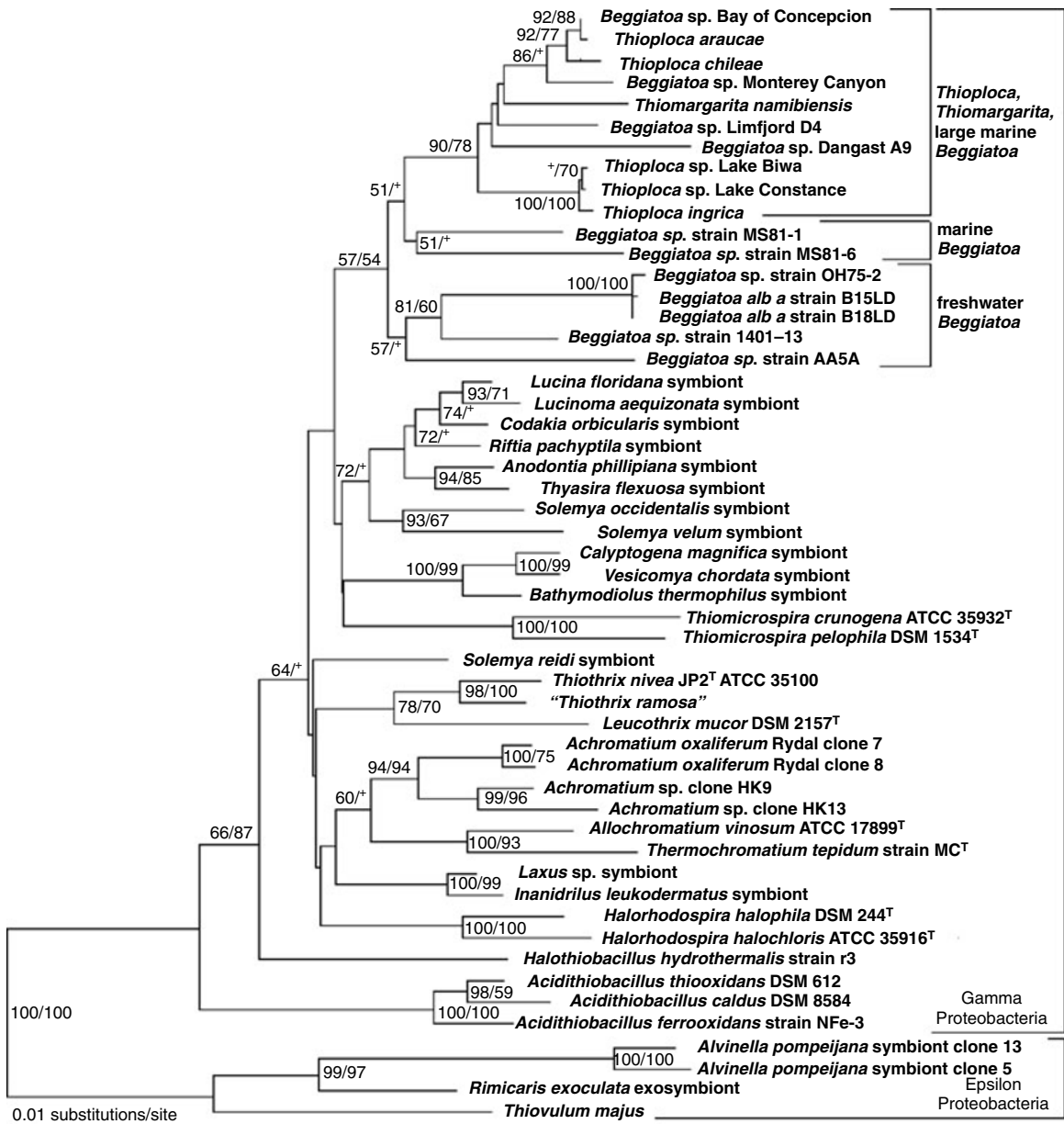


Fig. 16. Continued

tion of sulfide. These strategies are directly reflected in the structure of the mats and in the arrangement and the movements of the filaments within the oxic/anoxic gradient.

*Beggiatoa* and *Thioploca* are gradient organisms that occupy an ecological niche at the interface of sulfide as electron donor, and oxygen or nitrate as electron acceptor. Both genera have adapted to this niche in characteristic ways. The small freshwater and marine *Beggiatoa* spp. position themselves as a narrow layer at the oxygen/sulfide interface and separate the two compounds efficiently from each other. Sulfide is oxidized within the *Beggiatoa* mat and does not

diffuse into the oxic zone, at least not in high concentrations; oxygen is consumed within the *Beggiatoa* mat and does not reach the sulfidic layer below it (Jørgensen and Revsbech, 1983a; Møller et al., 1985; Nelson et al., 1986b; Fenchel and Bernard, 1995). The micro-oxic growth zone of *Beggiatoa* is characterized by oxygen concentrations in the range of 1–2.5 μM (Nelson et al., 1986b). This micro-oxic niche of *Beggiatoa* is created by highly dynamic sulfur-oxidizing metabolism of the *Beggiatoa* filaments themselves, but once established, it is remarkably stable. Laboratory gradient cultures remained active for several weeks and died only when the sulfide in the

bottom agar was exhausted (Nelson et al., 1986b).

A phobic response to high oxygen concentrations seems to be a driving force in establishing well-defined *Beggiatoa* mats. *Beggiatoa* filaments on a sediment surface adjust their position to short-term fluctuations in the sulfide and oxygen supply; they avoid high oxygen concentrations by contracting into the diffusive boundary layer directly at the sediment surface, and expand after the oxygen stress has passed (Møller et al., 1985). Oxidative damage to essential enzymes by peroxide formation is one of the presumed reasons for the general oxygen sensitivity of *Beggiatoa* spp.; for example, H<sub>2</sub>O<sub>2</sub> exposure inhibited fumarate hydratase, an essential TCA cycle enzyme, in the heterotrophically growing *Beggiatoa* freshwater strain D-405 (Grabovich et al., 1993). A cytochemical study using 3,3'-diaminobenzidine (DAB) showed the location of products of partial oxygen reduction, hydrogen peroxide and the superoxide radical. H<sub>2</sub>O<sub>2</sub> accumulated primarily in the periplasmic space, and on the outer surface of the cell wall; superoxide radical formation took place mostly at the cytoplasmic membrane with lesser amounts in the periplasmic space and on the outer cell wall surface (Chekanova and Dubinina, 1990). The rate of peroxide production was proportional to oxygen consumption during respiration; when electron donors (pyruvate, catalase, thiosulfate) were added to the culture medium, they acted as antioxidants which stabilized growth and increased the cell yields (Dubinina et al., 1990).

*Beggiatoa* can occur in association with other filamentous bacteria and form complex microbial mats, for example in benthic cyanobacterial mats (Garcia-Pichel et al., 1994). Cyanobacterial mats form a gradient habitat with suitable interface conditions for *Beggiatoa* (Jørgensen, 1982). At daytime, the upper layers of cyanobacterial mats are supersaturated with oxygen as a consequence of cyanobacterial photosynthesis. At night, oxygen production ceases and sulfide produced by sulfate reduction moves up towards the mat surface (Jørgensen et al., 1979; Jørgensen et al., 1983b). If this interface remains outside of the photic zone during the day, photosynthetic sulfur-oxidizing bacteria are excluded, and *Beggiatoa* spp. colonize a cyanobacterial mat and grow along the oxic/anoxic interface. The hypersaline cyanobacterial mats of Guerrero Negro are a good example (Jørgensen and DesMarais, 1986). Migrating *Beggiatoa* filaments closely follow the diel up-and-down movement of the oxygen/sulfide interface by ca. 1 mm, but always maintain a safe distance from fully oxic conditions (Garcia-Pichel et al., 1994). Their gliding speed of 1–2.6 µm per sec surpassed the speed of cyanobacteria in the same mat, which can retreat

into the mat matrix to avoid excessive irradiation during the day (Garcia-Pichel et al., 1994). A special case of cyanobacterial mats with a significant *Beggiatoa* component are the horizontally migrating, sulfide-producing and sulfide-oxidizing mats dominated by the gliding filamentous cyanobacterium *Phormidium corallyticum*. These mats are the causative agent of black-band coral disease, by overgrowing and completely degrading coral tissue (Carlton and Richardson, 1995).

The migrations of *Beggiatoa* filaments in microbial mats and cyanobacterially dominated sediments is not only regulated by the combined effects of oxygen and sulfide but is, in parallel, controlled by light. Experiments with *Beggiatoa* cultures from a warm freshwater spring (Hunter Spring) have shown a statistically significant photophobic response of individual *Beggiatoa* filaments, still detectable at low ambient light levels equivalent to ca. 2% of full summer sunlight intensity; filaments were most sensitive in the 400–500 nm spectrum that in nature is blocked out by cyanobacterial carotenoids. *Beggiatoa* filaments in field material maintained in the laboratory enrichments retreated into the sediment after short illumination, and returned to the sediment surface after several hours of darkness (Nelson and Castenholz, 1982a).

Large marine *Beggiatoa* spp. occupy a slightly different ecological niche than the small marine strains that were discussed previously; they are not tied to thin oxygen-sulfide interfaces but can tolerate fluctuating sulfide and oxidant levels. The extraordinary thickness of these mats (up to several cm), or their growth as virtual monocultures that can be harvested with a vacuum-cleaner-like device (Jannasch et al., 1989b; Nelson et al., 1989), rules out a growth pattern in the micrometer-thin interface of stabilized sulfide/oxygen gradients. Dense *Beggiatoa* mats growing on hydrothermally active sediments of the Guaymas Basin are exposed to irregularly fluctuating pulses of oxygenated seawater and sulfide from the sediment (Gundersen et al., 1992). This continuous mixing of sulfide and seawater is sustaining mats of extraordinary thickness. The ecophysiological flexibility of large *Beggiatoa* spp. is enhanced by their ability to accumulate nitrate intracellularly and to use it as electron acceptor for sulfide oxidation. All large *Beggiatoa* and *Thioploca* spp. studied so far accumulate nitrate from the 10–100 µM range in surrounding seawater to ca. 100 mM intracellular concentration (McHatton et al., 1996). For the Guaymas *Beggiatoa*, the intracellular nitrate accumulation is in the range of 50–100 mM (A. Teske, unpublished observation). These concentrations would not be possible with oxygen that reaches saturation around 250 µM. With this storage capacity,

*Beggiatoa* spp. can grow for several hours without external supply of oxidant (McHatton et al., 1996). The most likely nitrate reduction product, based on parallel results with *Thioploca* spp., is ammonia, not dinitrogen (Otte et al., 1999).

Large, nitrate-accumulating *Beggiatoa* spp. are not limited to hydrothermal vents and sulfide seeps but can be found on and within coastal, organic-rich, marine sediments as well (Jørgensen, 1977; Musmann et al., 2003). Nitrate-accumulating *Beggiatoa* spp. occur within anoxic, near-surface sediments where they oxidize sulfide produced in situ by sulfate reduction. Their nitrate-storing ability allows them to survive within the sediment, down to 2–4 cm depth. Thus, *Beggiatoa* spp. can occur within the sediment and do not always form conspicuous mats on the sediment surface (Musmann et al., 2003); in both cases, they play an important role in the nitrogen and sulfur cycle of marine sediments. A seasonal study of *Beggiatoa* mats in the Bay of Concepción showed how these seasonally developing *Beggiatoa* mats transform the bottom sediment from an ammonia sink to an ammonia source (Graco et al., 2001). Summer hypoxia at the bottom of the Bay favors the development of *Beggiatoa* spp. mats which reduce nitrate and release ammonia from the sediment into the water column, thus augmenting the traditional ammonia flux from anaerobic decomposition of organic matter. In parallel, sulfide buildup in the sediment porewater, due to increased organic matter degradation by sulfate reduction, inhibits nitrification and denitrification in the sediment and favors nitrate reduction by ammonia-producing *Beggiatoa* spp. (Graco et al., 2001). Similar findings have been reported for mats of large, vacuolated *Beggiatoa* spp. in Tokyo Bay (Sayama, 2001) and for *Beggiatoa* mats in coastal Danish sediments (Risgaard-Petersen, 1995). The ammonia released to the water column forms a nitrogen source for pelagic production; nitrogen loss by denitrification is reduced. By increasing benthic-pelagic coupling of ammonia, *Beggiatoa* mats increase local productivity and contribute to coastal eutrophication.

*Beggiatoa* mats also play a significant role for the benthic-pelagic exchange of sulfur. Sulfate-reducing bacteria, the source of biogenic sulfide at the sediment-water interface, are attracted to the top of the anoxic zone by the increased nutrient supply; many strains can to a limited degree respire with oxygen (Cypionka, 2001). Sediments of a shallow brackish fjord (Limfjorden, Denmark) showed the link between sulfate reduction and *Beggiatoa* population density. Both sulfate reduction rates and *Beggiatoa* biomass density peaked at the sediment surface, in the upper cm. The sulfide uptake of these *Beggiatoa* spp. was so efficient that sulfide accumu-

lated only below the *Beggiatoa* layer, and the sulfide concentrations within the *Beggiatoa* layer remained near zero (Musmann et al., 2003). In this way, the *Beggiatoa* filaments act as a sulfide trap that prevents toxic sulfide from entering the water column; during this process they enrich the sulfur content of surface sediments, and allow resuspension and recycling of partially oxidized sulfur species in the water column (Grant and Bathmann, 1987).

## Ecology of *Thioploca*

The ecology of *Thioploca* has been explored almost exclusively in the mats of large, nitrate-accumulating *Thioploca* species on the continental shelf off Chile and Peru (Fossing et al., 1995). The chemotactic motility that is characteristic for *Thioploca* was investigated with Chilean *Thioploca* species, and also the geochemical framework in the following discussion refers to the Chilean data, which give a multifaceted picture of the ecology of marine, nitrate-respiring *Thioploca*. That does not mean that other *Thioploca* spp. always behave similarly; the study of freshwater *Thioploca* spp. with modern approaches is just beginning (Kojima et al., 2003) and may yield surprises in the future.

The ecological niche of *Thioploca* is characteristically different from *Beggiatoa*. *Thioploca* is a gradient organism with a twist. *Thioploca* filaments can move up and down in their gelatinous sheaths that are embedded in the surface sediment. Thus, they can adjust their position in redox gradients by retreating into the sediment, or emerging through the benthic boundary layer and take up nitrate from the water column (Hüttel et al., 1996). Finely tuned phobic responses to high sulfide and high oxygen concentrations, and a strong attraction to nitrate that even overrides oxygen aversion to some degree, determine this complex behavior of *Thioploca*. After nitrate addition to seawater overlying a *Thioploca* mat, *Thioploca* filaments emerge partially from their sediment-embedded sheaths and sway in the current like white hair while taking up nitrate from the water (Hüttel et al., 1996). Subsequently, *Thioploca* filaments can retreat (at a speed of 3–5 mm h<sup>-1</sup>) into the sediment and use their stored nitrate for sulfide oxidation. In this way, *Thioploca* can bridge and exploit spatially separated pools of sediment sulfide and seawater nitrate and modify the “holding your breath” strategy of nitrate accumulation and respiration of large, marine *Beggiatoa* spp with limited motility. For large *Thioploca* spp. from the Chilean continental shelf, the intracellular nitrate and sulfur reserves allow a turnover time of 8–10 days (Otte et al., 1999). For longer-term survival, *Thioploca*

spp. require just the right balance of anoxia (nitrate, no oxygen) in the bottom water and sulfate-reducing activity in the sediment, in the sense that sulfide production must not overwhelm the sulfur-oxidizing capability of *Thioploca*. *Thioploca* is in fact very sensitive to changes in its environment. Temporary bottom water oxygenation during the southern winter depressed *Thioploca* populations significantly (Schulz et al., 2000). Also, local high sulfate reduction rates and high levels of free sulfide in the sediment changed the environmental balance from *Thioploca* mats to sheathless, *Beggiatoa*-like sulfur oxidizers on the sediment surface (Schulz et al., 2000).

This survival strategy of *Thioploca* spp. contrasts with the ecophysiology of its recently discovered relative *Thiomargarita namibiensis*. This immotile, giant sulfur oxidizer is the largest known prokaryote by volume; it relies completely on its enormous storage capacity for sulfur and nitrate to carry it through irregular natural fluctuations of sulfide and nitrate concentration in its sedimentary habitat (Schulz et al., 1999; Schulz, 2002a). In contrast to the very oxygen-sensitive Chilean *Thioploca* spp., *Thiomargarita namibiensis* tolerates prolonged oxygen exposure and, in addition to nitrate, appears to be able to use oxygen for sulfide oxidation if acetate is provided. This respiratory flexibility probably helps *Thiomargarita namibiensis* to tolerate irregular fluctuations of sulfide, nitrate and oxygen during resuspension episodes in its natural habitat (Schulz and DeBeer, 2002b).

The structure of the Chilean *Thioploca* mats is conducive to the microbial lifestyle of bridging sulfide and nitrate pools. The densest *Thioploca* mat matrix of randomly orientated filaments and

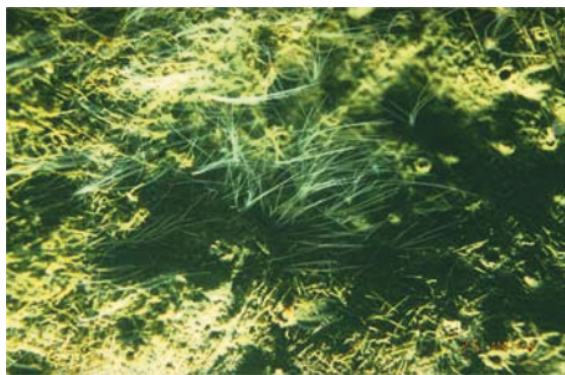


Fig. 17. A flume experiment with freshly harvested *Thioploca* mat from the Chilean continental shelf simulates in situ conditions where nitrate-accumulating *Thioploca* filaments extend 1–3 mm in a vertical direction above the sediment surface and through the diffusive boundary layer into the nitrate-rich current. From Hüttel et al. (1996).

bundles is found in the uppermost centimeter layer, while predominantly vertically orientated, less densely packed *Thioploca* bundles reach a depth of generally 4–8 cm, or maximally 10–15 cm (Schulz et al., 1996). The surface layer of the mat is generally well supplied with nitrate; it can penetrate several centimeters into the hydraulically conductive, porous and soft *Thioploca* mats sediments (Hüttel et al., 1996). The upper 1–5 cm of the sediment also show the highest sulfate reduction rates, up to 1500 nmol cm<sup>-3</sup> d<sup>-1</sup>, which are extremely high rates for marine sediments. Nevertheless, efficient in situ reoxidation of sulfide keeps the sulfide concentrations in the *Thioploca* mat sediments low, mostly in the range of 5–50 μm, and sulfate concentrations were never depleted below bottom water concentrations (Thamdrup and Canfield, 1996; Ferdelman et al., 1997). *Thioploca* mats contribute significantly to this in situ anaerobic sulfide reoxidation; different data sets and experimental approaches indicated a *Thioploca* contribution of 16–35%, or 25–91%, to the total in-situ sulfide oxidation in the sediment (Ferdelman et al., 1997; Otte et al., 1999).

This highly efficient internal sulfur recycling could benefit from the close spatial association between sulfate-reducing and sulfur-oxidizing bacteria in *Thioploca* mats. *Thioploca* biomass and cultivable most probable numbers (MPNs) of sulfate-reducing bacteria both peak in the surface layer of the mat (A. Teske, unpublished results). Filamentous sulfate reducers of the genus *Desulfonema* grow on and within the *Thioploca* sheaths, suggesting a complete cycle

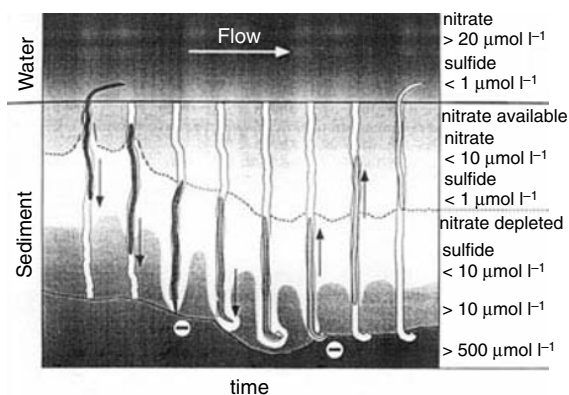


Fig. 18. Diagram showing how chemotactic responses and the concentration of an internal trigger may control vertical shuttling in *Thioploca* spp. The shading of the trichomes reflects the concentrations of the trigger (e.g., nitrate) in the filaments. The arrows indicate the chemotactic attraction of and the movement toward sulfide and nitrate, respectively. The minus signs indicate phobic responses to high concentrations of oxygen or sulfide. From Hüttel et al. (1996).

of sulfate reduction and reoxidation within a single *Thioploca* bundle (Fukui et al., 1999).

The high intracellular concentrations of sulfur and nitrate are correlated to the position and activity of *Thioploca* filaments in the gradient. “Deep” *Thioploca* filaments tend to consume their nitrate content for sulfide oxidation and build up sulfur globules, and replenish their nitrate stocks at the surface where sulfur is consumed (Zopfi et al., 2001). The resulting elemental composition of these nitrate- and sulfur-loaded organisms has brought *Thioploca* another nickname, “gunpowder bacteria” (L. P. Nielsen, personal communication).

Owing to nitrate reduction and ammonification by *Thioploca*, ammonia accumulates at high rates in *Thioploca*-harboring sediment surface layers (Thamdrup and Canfield, 1996). The *Thioploca* mats turn the sediments from a denitrifying nitrogen sink into an ammonia-producing nitrogen source; ammonia fluxes across the sediment-water interface constitute a major, readily utilized and recycled nitrogen source for the water column over *Thioploca*-harboring areas (Farias et al., 1996; Farias, 1998). A seasonal pattern for ammonia flux is likely, as shown by seasonal studies of the *Thioploca* and *Beggiatoa* mats within and near the Bay of Concepción (Schulz et al., 2000; Graco et al., 2001). The mats develop in austral spring and summer during upwelling of oxygen-depleted, nitrate-rich equatorial subsurface water, and decay in winter during downwelling of well-oxygenated sub-Antarctic surface water (Schulz et al., 2000; Graco et al., 2001). During austral summer, the *Beggiatoa* mats inside the Bay of Concepción turn the sediment into a net ammonia source (Graco et al., 2001); similar seasonal patterns of ammonia flux are likely for the *Thioploca* mats that dominate the shelf sediments outside of the Bay.

Thus, the Chilean *Thioploca* and *Beggiatoa* mats shape the nitrogen and sulfur cycles of their environment to a considerable degree. In general, *Thioploca* and *Beggiatoa* mats play a double environmental role as sulfide scrubbers and detoxifiers, and as nitrate-reducing ammonia producers that play an essential role in benthic-pelagic coupling of nitrogen sources.

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# The Family Halomonadaceae

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## Introduction

The family Halomonadaceae, within the Gammaproteobacteria, consists mostly of marine and moderately halophilic microorganisms that are phenotypically rather diverse. Because of this apparent lack of a core of differential phenotypic traits, many of its current species were previously assigned to other genera such as *Deleya* (now extinct), *Alcaligenes*, *Pseudomonas*, *Halovibrio*, *Volcaniella*, etc. Reorganizations among these species started by the mid 1990s with the aid of 16S rRNA gene sequence comparison. In the meanwhile, new descriptions within the family Halomonadaceae have been reported, and the increasing number of species led some authors to review its phylogeny (Arahal et al., 2002a) and phenotypic features (Mata et al., 2002).

A Subcommittee on the Taxonomy of the Halomonadaceae, a member of the International Committee on Systematic of Prokaryotes, was constituted recently (Vreeland and Ventosa, 2003) and can be taken as a sign of the increasing interest in this group of organisms.

The genera *Halomonas* and *Chromohalobacter* have been largely studied as model organisms of halophilism. Some of their representatives are among the most halophilic bacteria (Ventosa et al., 1998) and are adapted to a wide range of saline concentrations, even wider than extreme halophiles. Another source of interest for the study of this group of organisms has been their potential in biotechnological applications. These include the production of compatible solutes, extracellular enzymes (adapted to saline stress), and exopolysaccharides among others.

## Phylogeny

Five main phylogenetic studies have been conducted on the Halomonadaceae. In the first one (Franzmann et al., 1988), which was the basis for

the proposal of this family, the method employed was the 16S rRNA oligonucleotide cataloguing technique. Later, Dobson et al. (1993) obtained the 16S rRNA sequences of *Halomonas (Deleya) aquamarina*, *Halomonas (Deleya) halophila*, *Halomonas (Deleya) marina*, *Halomonas elongata*, *Halomonas meridiana*, *Halomonas (Halovibrio) variabilis* and *Halomonas subglaciescola* (sequence accession numbers [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93352\[M93352\]](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93352[M93352]), [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93353\[M93353\]](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93353[M93353]), [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93354\[M93354\]](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93354[M93354]), [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93355\[M93355\]](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93355[M93355]), [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93356\[M93356\]](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93356[M93356]), [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93357\[M93357\]](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93357[M93357]), and [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93358\[M93358\]](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=M93358[M93358])) and analyzed them together with the sequences of *Halomonas halmophila* and other species belonging to the Gammaproteobacteria. They showed that the level of similarity among the sequences of members of the family Halomonadaceae is 92.6–100% and that the phylogenetic grouping did not correspond to the taxonomic assignment of the species analyzed, suggesting the unification into a single genus. They also proposed a number of characteristic sequence signatures of the members of the family Halomonadaceae that have been readapted in other studies (Dobson and Franzmann, 1996; Arahal et al., 2002a), as new members have been described. However, full-sequence analyses are much more informative than signatures alone since the latter have to be redefined on the basis of present and future new species.

Mellado et al. (1995b) conducted a phylogenetic study on six new 16S rRNA sequences ({X87217}, {X87218}, {X87219}, {X87220}, {X87221}, and {X87222}), corresponding to

*Chromohalobacter marismortui* (four strains), *Halomonas* (*Volcaniella*) *eurihalina*, *Halomonas* (*Deleya*) *salina*, and close relatives. They proposed the reclassification of *Volcaniella eurihalina* as *Halomonas eurihalina* but highlighted the need of a polyphasic approach to determine the natural taxonomic position of members of the Halomonadaceae, especially because their heterogeneity was (and still is) too large for a single genus.

Further studies by Dobson and Franzmann (1996) determined another seven 16S rRNA sequences ({L42614}, {L42615}, {L42616}, {L42617}, {L42618}, {L42619}, and {L42620}) corresponding to the type strains of *Halomonas subglaciescola*, *Halomonas* (*Deleya*) *cupida*, *Halomonas* (*Deleya*) *pacifica*, *Halomonas* (*Deleya*) *salina*, *Halomonas* (*Deleya*) *venusta*, *Halomonas halodurans* and *Halomonas* (*Volcaniella*) *eurihalina*. On the basis of their results, they proposed the unification of the genera *Deleya*, *Halomonas* and *Halovibrio* and the species *Paracoccus halodenitrificans* into the genus *Halomonas*.

Recently, Arahal et al. (2002a) evaluated the phylogenetic status of the family Halomonadaceae using 16S and 23S rRNA sequences. In addition to the new sequences determined in their study, 18 for the 23S rRNA (AJ306870, AJ306871, AJ306872, AJ306873, AJ306874, AJ306875, AJ306876, AJ306877, AJ306878, AJ306879, AJ306880, AJ306881, AJ306882, AJ306883, AJ306884, AJ306885, AJ306886, and AJ306887) and 7 for the 16S rRNA (AJ306888, AJ306889, AJ306890, AJ306891, AJ306892, AJ306893, and AJ306894), the sequences were compared to more than 16,000 full or almost full rRNA sequences. By that time, the number of those sequences that could be ascribed to the family Halomonadaceae exceeded 70 (including many sequences from environmental clones and poorly characterized isolates). In addition, several treeing methods were used to elucidate the most stable branchings. A good agreement between the 16S rRNA- and the 23S rRNA-derived trees was obtained. According to this study, the genus *Halomonas* was formed by two well-defined phylogenetic groups (containing five and seven species, respectively) as well as six species that could not be assigned to any of the above-mentioned groups. Group 1 comprised *Halomonas elongata* (type species of the genus), *Halomonas eurihalina*, *Halomonas halmophila*, *Halomonas halophila* and *Halomonas salina*, all bearing a 98.2% average sequence (16S rRNA or 23S rRNA) similarity. Group 2 included the species *Halomonas aquamarina*, *Halomonas meridiana*, *Halomonas magadiensis*, *Halomonas variabilis*, *Halomonas venusta*, *Halomonas halo-*

*durans* and *Halomonas subglaciescola*, and exhibited a 97.6% mean 23S rRNA sequence similarity (97.4% in the case of the 16S rRNA sequences). The species *Halomonas pacifica*, *Halomonas halodenitrificans*, *Halomonas cupida*, *Halomonas desiderata*, *Halomonas campisalis* and *Halomonas pantelleriensis*, not only do not clearly fall into either of the two groups mentioned above but also shared relatively low values of sequence similarity with them or even between themselves (91.7–96.7%; Arahal et al., 2002a). The phylogenetic data are in agreement with the phenotypic heterogeneity reported for the species of the genus *Halomonas* or the wide range of G+C content (52–68 mol%) described for the different species of *Halomonas*.

With respect to the genus *Chromohalobacter*, the four species described within this genus formed a group closely related to *Halomonas*. The average rRNA sequence similarity of species of *Chromohalobacter* is 98.6% (for the 23S rRNA) and 98.5% (for the 16S rRNA). Within this group falls the sequence of *Pseudomonas beijerinckii*, an organism probably misclassified. If their sequences are compared to those of other halomonads, values below 95% (generally accepted as a good borderline for genus separation) are obtained in all cases. Similar low values were obtained for the sequence of *Halomonas marina*, which forms a deeper branch of the *Halomonas-Chromohalobacter* group. Indeed, according to this and other data, this organism was proposed as the type species of a new genus, named “*Cobetia*” (Arahal et al., 2002b). Finally, the sequences of *Zymobacter palmae* and *Carnimonas nigrificans* show a deeper branching in the tree (Fig. 1). Their 16S rRNA sequence similarity is 93.5% and even lower values are obtained when comparing any of the two with the other members of the family.

In the meanwhile, five new species have been proposed within the genus *Halomonas*: *Halomonas alimentaria* (its closest relative is *H. halodenitrificans*; Yoon et al., 2002), *Halomonas halocynthiae* (which cannot be ascribed to any of the rRNA groups of *Halomonas*; Romanenko et al., 2002), *Halomonas marisflavi* (whose branch falls between that of *Cobetia marina* and the branch of *Zymobacter palmae* and *Carnimonas nigrificans*; Yoon et al., 2001), *Halomonas maura* (which falls into rRNA group 1 of the *Halomonas*; Bouchotroch et al., 2001), and *Halomonas muralis* (its closest relative is *H. pantelleriensis*; Heyrman et al., 2002; Fig. 1).

From the phylogenetic point of view, some well-defined relationships can be observed within members of the Halomonadaceae. These groups, as defined above, are stable regardless of the methodology employed. Other relations may

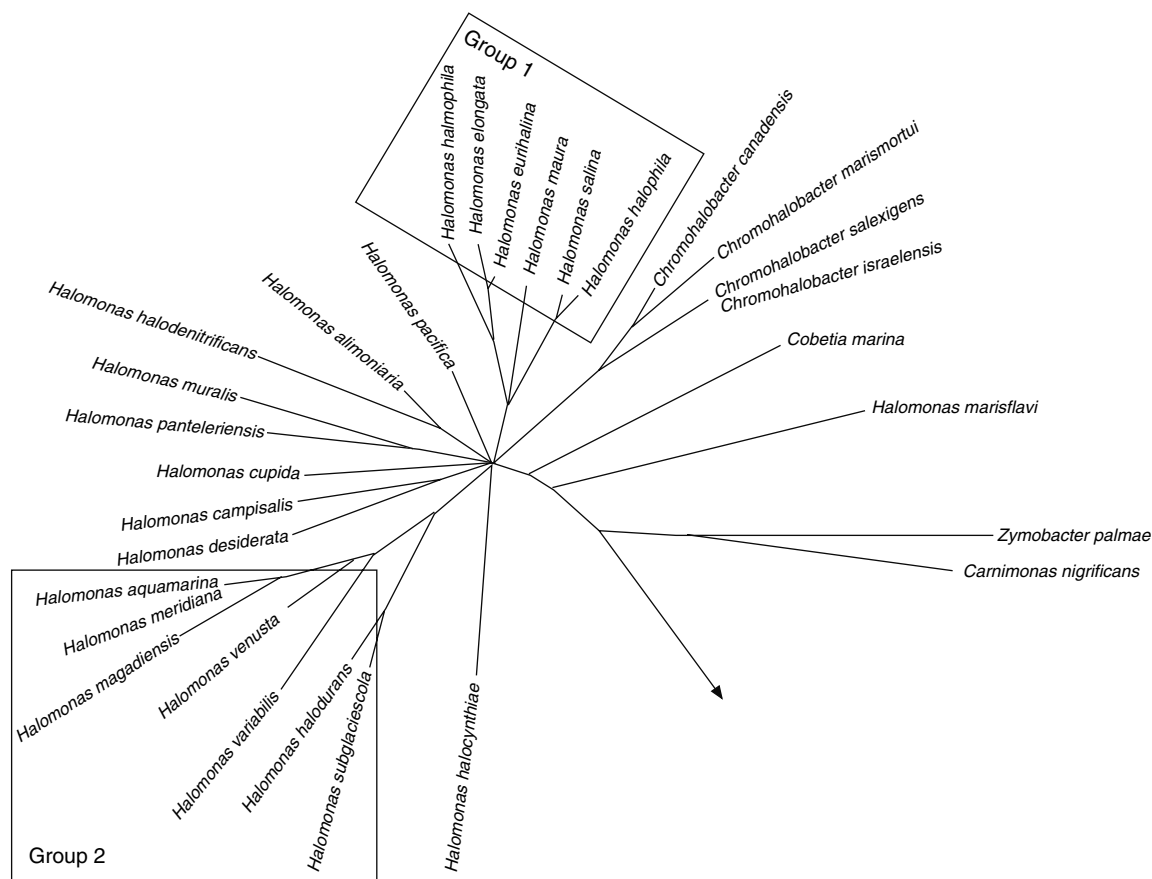


Fig. 1. 16S rDNA based tree of members of the family Halobacteriaceae. An explanation about the methodology followed for sequence analysis can be found in Arahal et al. (2002a). The arrow points to the outgroup, which has been removed to simplify the figure. Nodes with more than two branches indicate that their relative order could not be elucidated comparing different treeing methods. *Halomonas* species that can be ascribed to the 16S rRNA groups 1 and 2 of Arahal et al. (2002a) have been marked accordingly. Bar, 2% estimated sequence divergence.

become better defined once more in-between sequences become available.

## Taxonomy

The family Halomonadaceae belongs, together with the “Alcanivoraceae,” and the “Oceanospirillaceae,” to the “Oceanospirillales,” an order within the Gammaproteobacteria (Garrity et al., 2003) that consists mainly of marine species.

The family Halomonadaceae was originally proposed by Franzmann et al. (1988) and it was later emended by Dobson and Franzmann (1996). At the start of 2004, this family included five recognized genera: *Halomonas* (type genus), *Carnimonas*, *Chromohalobacter*, *Cobetia* and *Zymobacter* (Garrity et al., 2003). Table 1 contains relevant taxonomic information on these genera and their species.

As mentioned before, some of the current species were isolated and described many years before the proposal of the genera *Halomonas* (Vreeland et al., 1980b), *Chromohalobacter* (Ventosa et al., 1989) or *Cobetia* (Arahal et al., 2002b): “*Chromobacterium marismortui*” (now *Chromohalobacter marismortui*), “*Arthrobacter marinus*” (earlier synonym of *Cobetia marina*), “*Achromobacter aquamarinus*” (*Halomonas aquamarina*), *Flavobacterium halmophilum* (basonym of *Halomonas halmophila*) or “*Micrococcus halodenitrificans*” (*Halomonas halodenitrificans*) are the oldest examples. In 1972, Baumann and coworkers published an extensive taxonomic study of Gram-negative, nonfermentative marine bacteria, including four organisms assigned at that time to the genus *Alcaligenes*, namely *Alc. aestus*, *Alc. cupidus*, *Alc. pacificus* and *Alc. venustus*. About one decade later, Baumann et al. (1983) proposed the creation of the

Table 1. Genera and species of the family Halomonadaceae.

Genus and species name	Type species and type strain	References
<i>Carnimonas</i>	<i>Carnimonas nigrificans</i>	Garriga et al., 1998
<i>Carnimonas nigrificans</i>	CTCBS1 = ATCC BAA-78 = CECT 4437 = CIP 105703	Garriga et al., 1998
<i>Chromohalobacter</i>	<i>Chromohalobacter marismortui</i>	Ventosa et al., 1989
<i>Chromohalobacter canadensis</i>	ATCC 43984 = CECT 5385 = CCM 4919 = CIP 105571 = DSM 6769 = LMG 19547 = NCIMB 13767	Arahal et al., 2001a Huval et al., 1995
Basonym: <i>Halomonas canadensis</i>		
<i>Chromohalobacter israelensis</i>	Ba1 = ATCC 43985 = CECT 5287 = CCM 4920 = CIP 106853 = DSM 6768 = LMG 19546 = NCIMB 13766	Arahal et al., 2001a Huval et al., 1995
Basonym: <i>Halomonas israelensis</i>		
<i>Chromohalobacter marismortui</i>	CCM 3518 = ATCC 17056 = DSM 6770 = LMG 3935	Ventosa et al., 1989 Elazari-Volcani, 1940
Synonym: "Chromobacterium marismortui"		
<i>Chromohalobacter salexigens</i>	ATCC BAA-138 = CECT 5384 = CCM 4921 = CIP 106854 = DSM 3043 = NCIMB 13768	Arahal et al., 2001b
<i>Cobetia</i>	<i>Cobetia marina</i>	Arahal et al., 2002b
<i>Cobetia marina</i>	219 = ATCC 25374 = CECT 4278 = CIP 104765 = DSM 4741 = LMG 2217 = NCIMB 1877	Arahal et al., 2002b Baumann et al., 1972 Baumann et al., 1983 Dobson and Franzmann, 1996 Cobet et al., 1970
Basonym: <i>Pseudomonas marina</i>		
Other synonyms: <i>Deleya marina</i> <i>Halomonas marina</i> "Arthrobacter marinus"		
<i>Halomonas</i>	<i>Halomonas elongata</i>	Vreeland et al., 1980b
<i>Halomonas alimentaria</i>	YKJ-16 = KCCM 41042 = JCM 10888	Yoon et al., 2002
<i>Halomonas aquamarina</i>	558 = ATCC 14400 = CCUG 16157 = CIP 105454 = DSM 30161 = IAM 12550 = LMG 2853 = NCIMB 557	Dobson and Franzmann, 1996 Hendrie et al., 1974 Akagawa and Yamasato, 1989 ZoBell and Upham, 1944 Krasil'nikov, 1949
Basonym: <i>Alcaligenes aquamarinus</i>		
Other synonyms: <i>Deleya aquamarina</i> , "Achromobacter aquamarinus" "Bacterium aquamarinum"		
<i>Halomonas campisalis</i>	A4 = ATCC 700597 = CIP 106639	Mormile et al., 1999
<i>Halomonas cupida</i>	79 = ATCC 27124 = CCUG 16075 = CIP 103199 = DSM 4740 = LMG 3448	Dobson and Franzmann, 1996 Baumann et al., 1972 Baumann et al., 1983
Basonym: <i>Alcaligenes cupidus</i>		
Other synonym: <i>Deleya cupida</i>		
<i>Halomonas desiderata</i>	FB2 = CIP 105505 = DSM 9502 = LMG 19548	Berendes et al., 1996
<i>Halomonas elongata</i>	1H9 = ATCC 33173 = CIP 104264 = DSM 2581 = IFO15536 = LMG 9076	Vreeland et al., 1980b
<i>Halomonas eurihalina</i>	F9-6 = ATCC 49336 = CIP 106091 = DSM 5720	Mellado et al., 1995b Quesada et al., 1990
Basonym: <i>Volcaniella eurihalina</i>		
<i>Halomonas halmophila</i>	ACAM 71 = ATCC 19717 = CIP 105455 = DSM 5349 = IFO15537 = LMG 4023 = NCMB 1971	Franzmann et al., 1988 Dobson et al., 1990 Elazari-Volcani, 1940
Basonym: <i>Flavobacterium halmophilum</i>		
<i>Halomonas halocynthiae</i>	DSM 14573 = KMM 1376	Romanenko et al., 2002
<i>Halomonas halodenitrificans</i>	ATCC 13511 = CIP 105456 = DSM 735 = IFO14912	Dobson and Franzmann, 1996 Davis, 1969 Robinson and Gibbons, 1952
Basonym: <i>Paracoccus halodenitrificans</i>		
Other synonym: "Micrococcus halodenitrificans"		
<i>Halomonas halodurans</i>	ATCC 29686 = DSM 5160 = IFO15607	Hebert and Vreeland, 1987 Rosenberg, 1983
Basonym: "Pseudomonas halodurans"		
<i>Halomonas halophila</i>	F5-7 = ATCC 49969 = CCM 3662 = CIP 103512 = DSM 4770 = LMG 6456	Dobson and Franzmann, 1996 Quesada et al., 1984
Basonym: <i>Deleya halophila</i>		
<i>Halomonas magadiensis</i>	21 MI = CIP 106823 = CIP 106874 = NCIMB 13595	Duckworth et al., 2000
<i>Halomonas marisflavi</i>	SW32 = CIP 107103 = JCM 10873 = KCCM 80003	Yoon et al., 2001
<i>Halomonas maura</i>	S-31 = ATCC 700995 = CECT 5298 = DSM 13445	Bouchotroch et al., 2001



Table 1. *Continued*

Genus and species name	Type species and type strain	References
<i>Halomonas meridiana</i>	ACAM 246 = ATCC 49692 = CIP 104043 = DSM 5425 = IFO15608 = UQM 3352	James et al., 1990
<i>Halomonas muralis</i>	DSM 14789 = LMG 20969	Heyrman et al., 2002
<i>Halomonas pacifica</i>	62 = ATCC 27122 = CIP 103200 = DSM 4742 = LMG 3446	Dobson and Franzmann, 1996 Baumann et al., 1972 Baumann et al., 1983
Basonym: <i>Alcaligenes pacificus</i>		
Other synonym: <i>Deleya pacifica</i>		
<i>Halomonas pantelleriensis</i>	AAP = ATCC 700273 = CIP 105506 = DSM 9661 = LMG 19550	Romano et al., 1996
<i>Halomonas salina</i>	F8-11 = ATCC 49509 = CIP 106092 = DSM 5928	Dobson and Franzmann, 1996 Valderrama et al., 1991
Basonym: <i>Deleya salina</i>		
<i>Halomonas subglaciaccola</i>	ACAM 12 = ATCC 43668 = CIP 104042 = DSM 4683 = IFO 14766 = LMG 8824 = UQM 2926	Franzmann et al., 1987
<i>Halomonas variabilis</i>	III = ATCC 49240 = CIP 105504 = DSM 3051	Dobson and Franzmann, 1996 Fendrich, 1989
Basonym: <i>Halovibrio variabilis</i>		
<i>Halomonas venusta</i>	86 = ATCC 27125 = CCUG 16063 = CIP 103201 = DSM 4743 = LMG 3445	Dobson and Franzmann, 1996 Baumann et al., 1972 Baumann et al., 1983
Basonym: <i>Alcaligenes venustus</i>		
Other synonym: <i>Deleya venusta</i>		
<i>Zymobacter</i>	<i>Zymobacter palmae</i>	Okamoto et al., 1993
<i>Zymobacter palmae</i>	T109 = ATCC 51623 = DSM 10491 = IAM 14233	Okamoto et al., 1993

Abbreviations: ATCC, American Type Culture Collection, Manassas, Virginia, USA; CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Burjasot, Spain; CIP, Institut Pasteur, Paris, France; CCM, Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMG, Universiteit Gent, Laboratorium voor Mikrobiologie, Gent, Belgium; NCIMB, National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Aberdeen, Scotland, United Kingdom; KCCM, Korean Culture Center of Microorganisms, Department of Food Engineering, Yonsei University, Seoul, Republic of Korea; JCM, Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Hiroshima, Wako-shi, Japan; CCUG, Culture Collection, University of Göteborg, Dept. of Clinical Bacteriology, Göteborg, Sweden; KMM, Collection of Marine Microorganisms, Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia; IFO, Institute for Fermentation, Osaka, Japan; ACAM, Australian Collection of Antarctic Microorganisms, Hobart, Australia; and IAM, Institute of Applied Microbiology, University of Tokyo, Institute of Molecular and Cellular Bioscience, Tokyo, Japan.

genus *Deleya* to accommodate those four marine species as well as *Pseudomonas marina*.

The genera *Halomonas* and *Deleya* served as the basis for the creation of the family Halomonadaceae (Franzmann et al., 1988). At that time these genera contained four and six species, respectively.

A chemotaxonomic study (Franzmann and Tindall, 1990) of members of the family Halomonadaceae concluded that on the basis of respiratory quinone, polar lipid, and fatty acid compositions, no clear distinction existed at the genus level. Only a few months earlier Ventosa et al. (1989) proposed *Chromohalobacter* as a new genus with a single species, *C. marismortui*, on the basis of a subculture of "*Chromobacterium marismortui*," isolated from the Dead Sea (Elazari-Volcani, 1940), and seven moderately halophilic isolates from a Mediterranean saltern in Spain that were found to be very closely related to it. Later, in the

phylogenetic study of Mellado et al. (1995b) it was concluded that this genus belongs to the family Halomonadaceae.

The genus *Zymobacter*, with its single species *Z. palmae*, was created by Okamoto et al. (1993) and placed later in the family Halomonadaceae (Dobson and Franzmann, 1996).

By then, 16S rRNA phylogenetic analyses were used as definitive evidence of the lack of correlation in the taxonomic arrangements within the family Halomonadaceae (Dobson et al., 1993). Mellado et al. (1995b) proposed the reclassification of *Volcaniella eurihalina* as *Halomonas eurihalina* and pointed out the heterogeneity of the *Halomonas-Deleya* complex. Dobson and Franzmann (1996) transferred all species of the genus *Deleya* to the genus *Halomonas* together with *Halovibrio variabilis* and *Paracoccus halodenitrificans*. In one way, this simplification stopped the confusion of the naming within the *Halomonas-Deleya* complex, but

the resulting genus, *Halomonas*, contained (and still does) very different species and it is considered too heterogeneous. The genus *Halomonas* was expanded to 15 species, with few characters in common, while the only two other genera recognized at that time, *Chromohalobacter* and *Zymobacter*, contained one each. Meanwhile, the genus *Carnimonas* was created by Garriga et al. (1998) to accommodate one single species, *C. nigrificans* isolated from cured meat products, and later it was included into the family Halomonadaceae (Arahal et al., 2002a).

In recent years the genus *Halomonas* continued to include new species since new descriptions were carried out. As of January 2004 there are 23 validly published species names. These do not include *Halomonas canadensis* and *Halomonas israelensis* (Arahal et al., 2001a), which were transferred to the genus *Chromohalobacter*, or *Halomonas marina*, which was proposed as *Cobetia marina* (Arahal et al., 2002b). The list of articles in press of the International Journal of Systematic and Evolutionary Microbiology (<http://intl-ij.sgmjournals.org/misc/pip.shtml>) include the proposals of several new *Halomonas* and *Chromohalobacter* species for which the following names have been proposed: *H. neptunia*, *H. sulfidaeris*, *H. axialensis*, *H. hydrothermalis*, *H. boliviensis*, *H. ventosae*, *H. anticariensis*, *H. organivorans*, *C. sarecenensis*, *H. koreensis*.

A valuable effort to address the taxonomy of the group from a different perspective than the prevailing one (that is, ribosomal RNA phylogeny), is the study of Mata et al. (2002). In this article, they present a detailed phenotypic characterization of the type strains of all *Halomonas* species recognized at that time and the intraspecific variation of four of those species by studying 87 additional strains. The authors compared the reactions of 234 morphological, physiological, biochemical, nutritional and antimicrobial susceptibility tests. Part of the nutritional characterization was obtained by using a miniaturized (Biolog) identification system. It was the first time that such a method was employed so extensively among halomonads. In addition to the new data that were presented in their paper, some differences were observed between their results and those from the original species descriptions. Numerical analyses demonstrated the phenotypic heterogeneity of the *Halomonas* species (Mata et al., 2002). An important conclusion of the study of Mata et al. (2002) is that phenotypic traits can be selected according to their usefulness for distinguishing *Halomonas* species. It would be desirable that future taxonomic proposals within the genus *Halomonas* reach the same level of completeness in their descriptions and that such studies

be extended to the other genera of the family Halomonadaceae.

## Habitat

According the original description of the family Halomonadaceae (Franzmann et al., 1988), its members typically occurred in “temperate and Antarctic saline lakes, solar salt facilities, saline soils and marine environments.” This is still true for the majority of the current species (Table 2). Indeed the only exceptions to the above definition are *Carnimonas nigrificans*, *Chromohalobacter canadensis*, *Halomonas alimentaria*, *Halomonas desiderata*, *Halomonas halodenitrificans*, *Halomonas muralis* and *Zymobacter palmae*. Of these seven organisms, only *Zymobacter palmae* is neither halophilic nor halotolerant (however, it is very tolerant to ethanol—up to 6%).

So, the halomonads can be found in any saline environment, regardless of its geographical location. This includes oceans and seas (even at considerable depths), saline soils, salty foods, naturally occurring saline lakes, solar pans, etc. Since some of its members are also alkaliphilic, they are found in soda lakes and alkaline soils.

On the genus level, not surprisingly, *Halomonas* is found to be the most ubiquitous genus with the largest number of species, and these are very heterogeneous.

As for the interactions of halomonads with other microorganisms, Ivanova et al. (2002) characterized a heterotrophic microbial enrichment community established during the degradation of brown algae *Fucus evanescens*, and consisting of two species, *Pseudoalteromonas* sp. and *C. marina*. While the first was highly metabolically active (14 hydrolytic activities could be detected) and likely plays the main role in the initial stages of algal degradation, the second, *C. marina*, produced only caseinase and DNase but was resistant to the bacteriolytic activity of the former and utilized the degradation products of polysaccharides.

More recently, in a study about the temporal stability and biodiversity of two complex antilisterial cheese-ripening microbial consortia (Maoz et al., 2003), out of 400 isolates, three were identified as *H. venusta*, two as *H. variabilis*, and two as *Halomonas* sp. by Fourier-transform infrared spectroscopy and 16S ribosomal RNA sequence analysis.

## Isolation

Halomonads may be isolated following standard microbiological techniques on complex or

Table 2. Habitats of members of the family Halomonadaceae from which the original strains were isolated.

Species	Habitat
<i>Carnimonas nigrificans</i>	Raw cured meat products
<i>Chromohalobacter canadensis</i>	Contaminant on medium containing 25% NaCl
<i>Chromohalobacter israelensis</i>	Dead Sea
<i>Chromohalobacter marismortui</i>	Dead Sea and salterns
<i>Chromohalobacter salexigens</i>	Salterns
<i>Cobetia marina</i>	Marine environment
<i>Halomonas alimentaria</i>	Jeotgal (Korean traditional fermented seafood)
<i>Halomonas aquamarina</i>	Marine environment
<i>Halomonas campisalis</i>	Soil below a crystalline salt surface
<i>Halomonas cupida</i>	Marine environment
<i>Halomonas desiderata</i>	Municipal sewage
<i>Halomonas elongata</i>	Salterns
<i>Halomonas eurihalina</i>	Hypersaline habitats (soils and salterns) and seawater
<i>Halomonas halmophila</i>	Dead Sea
<i>Halomonas halocynthiae</i>	Gill tissues of the ascidian <i>Halocynthia aurantium</i>
<i>Halomonas halodenitrificans</i>	Meat-curing brines
<i>Halomonas halodurans</i>	Estuarine water
<i>Halomonas halophila</i>	Saline soils
<i>Halomonas magadiensis</i>	Littoral sediments of saline and alkaline East African soda lakes
<i>Halomonas marisflavi</i>	Marine environment
<i>Halomonas maura</i>	Saltern
<i>Halomonas meridiana</i>	Antarctic saline lakes
<i>Halomonas muralis</i>	Biofilm covering a wall and a mural
<i>Halomonas pacifica</i>	Marine environment
<i>Halomonas pantelleriensis</i>	Hard sand
<i>Halomonas salina</i>	Hypersaline soils, salt ponds, salt lakes, and seawater
<i>Halomonas subglaciescola</i>	Antarctic saline lake (Organic Lake).
<i>Halomonas variabilis</i>	North Arm of the Great Salt Lake, Utah (USA)
<i>Halomonas venusta</i>	Marine environment
<i>Zymobacter palmae</i>	Palm sap in Okinawa Prefecture (Japan)

defined media with a suitable salinity and incubated at room temperature for 1–7 days.

Since not all species show the same requirements (or tolerance) of salinity (Table 3), differences are expected to occur depending on the final salt content of the media. With the only exception of *Zymobacter palmae*, all species are able to grow at salinities in the range of 3.5–10% but not necessarily at their optimum over the whole range. Media for the selective isolation of moderately halophilic bacteria can be prepared with higher salt contents (for instance 20%) to prevent the growth of non-adapted competitors. The selectivity of such media can be increased by lowering the concentration of Mg<sup>2+</sup> to prevent the growth of extreme halophiles (Ventosa et al., 1982). However, not all the species within the family Halomonadaceae are able to grow at such high salinities.

The growth of species of *Chromohalobacter* and some *Halomonas* such as *H. aquamarina*, *H. cupida*, *H. eurihalina*, *H. halmophila*, *H. halodurans*, *H. halophila*, *H. maura*, *H. pantelleriensis*, *H. subglaciescola* and *H. variabilis* will be favored in media with a salt content of 7.5–10%. Some of them will yield reasonable growth at

15% salts or even 20%. At these concentrations, growth of extremely halophilic Archaea can occur, but they are easily distinguished by their red pigmentation. Most of the above-mentioned species have a minimum requirement for NaCl of 1–3.5%.

Other species (*Cobetia marina*, *H. alimentaria*, *H. campisalis*, *H. elongata*, *H. halodenitrificans*, *H. marisflavi*, *H. muralis* and *H. salina*) are also moderately halophilic, but their optimum salinity for growth is lower (around 5%). In contrast to the previous group, most of these species show no requirement of salts or require only as little as 0.5%.

The slightly halophilic species (*H. desiderata*, *H. halocynthiae*, *H. magadiensis*, *H. meridiana*, *H. pacifica* and *H. venusta*) grow optimally at around 3% NaCl. However, these species are halotolerant of up to 20% NaCl (15% in the case of *H. halocynthiae*).

*Carnimonas nigrificans* shows only a limited level of halotolerance. For its isolation, two selective media have been proposed (Garriga et al., 1998), cetrimide agar and MacConkey agar, although it may grow in other media such as tryptone soy agar. Although the organism is not

Table 3. Physiological features of members of the family Halomonadaceae.

Species	Salt range (% (w/v))		Temperature (°C)	pH
	minimum	optimum-maximum	minimum-maximum	minimum-maximum
<i>Carnimonas nigrificans</i>	0	4–8	nd	nd
<i>Chromohalobacter canadensis</i>	3	7.5–25	15–45	5.0–9.0
<i>Chromohalobacter israelensis</i>	3.5	8–20	15–45	5.0–9.0
<i>Chromohalobacter marismortui</i>	2	10–30	5–45	5.0–10.0
<i>Chromohalobacter salexigens</i>	0.9	7.5–10–25	15–45	5.0–9.0
<i>Cobetia marina</i>	0.5	5–20	10–42	5.0–10.0
<i>Halomonas alimentaria</i>	nd	1–13–23	4–45	5.0–nd
<i>Halomonas aquamarina</i>	0.5	7.5–10–20	15–37	5.0–10.0
<i>Halomonas campisalis</i>	0.5	5–15	4–50	8.0–11.0
<i>Halomonas cupida</i>	0	7.5–10–15	15–37	5.0–10.0
<i>Halomonas desiderata</i>	0	1–5–20	10–45	7.0–11.0
<i>Halomonas elongata</i>	0	3–8–20	4–45	5.0–10.0
<i>Halomonas eurihalina</i>	0.5	7.5–25	4–45	5.0–10.0
<i>Halomonas halmophila</i>	3	7.5–25	15–45	5.0–10.0
<i>Halomonas halocynthiae</i>	0.5	3–6–15	7–35	5.0–11.0
<i>Halomonas halodenitrificans</i>	3	5–9–20	5–37	5.0–10.0
<i>Halomonas halodurans</i>	3	8–20	4–37	5.0–10.0
<i>Halomonas halophila</i>	2	7.5–25	4–45	5.0–10.0
<i>Halomonas magadiensis</i>	0	0–7–20	20–45	5.0–11.0
<i>Halomonas marisflavi</i>	0.5	0.5–12–27	4–37	5.0–10.0
<i>Halomonas maura</i>	1	9–20	10–45	5.0–10.0
<i>Halomonas meridiana</i>	0	1–3–20	4–45	5.0–10.0
<i>Halomonas muralis</i>	0	2.5–10–15	10–35	5.5–10.0
<i>Halomonas pacifica</i>	0	0.5–3–20	4–45	5.0–10.0
<i>Halomonas pantelleriensis</i>	1	10–15	10–45	6.0–11.0
<i>Halomonas salina</i>	2	5–20	4–45	5.0–10.0
<i>Halomonas subglaciescola</i>	0.5	5–10–20	0–45	5.0–10.0
<i>Halomonas variabilis</i>	1	10–25	15–37	6.0–9.0
<i>Halomonas venusta</i>	0	0.5–7–20	4–45	5.0–10.0
<i>Zymobacter palmae</i>		nd	21–39	3.0–10.0

Abbreviation: nd, not determined.

pigmented, it produces black spots on the surface of cured meat products (the isolation source). This coloration seems to be the consequence of nonenzymatic reactions of substances derived from the meat surface.

Finally, *Zymobacter palmae* is neither halophilic nor halotolerant, but other special features of this organism can be used for its isolation. Thus, Okamoto et al. (1993) first selected for ethanol-tolerant bacteria using media with 5% (v/v) ethanol and then tested isolates for production of ethanol from maltose. *Zymobacter palmae* was found to produce ethanol from the fermentation of a variety of sugars: hexoses,  $\alpha$ -linked di- and tri-saccharides, and sugar alcohols (fructose, galactose, glucose, mannose, maltose, melibiose, saccharose, raffinose, mannitol and sorbitol).

Regarding pH, most species are neutrophilic and therefore the pH of media can be adjusted to around 7.2–7.5. Exceptions to this rule are *H. campisalis*, *H. desiderata*, *H. magadiensis* and *H.*

*pantelleriensis* that grow optimally at pH 9.0 or 9.5 (Table 3).

Finally, although all halomonads are mesophilic, some differences in their behavior towards temperature can be used for selective isolation. *Chromohalobacter marismortui*, *C. salexigens*, *C. marina*, *H. desiderata*, *H. elongata*, *H. halophila* and *H. magadiensis* grew well at 37°C while the rest grew only suboptimally (or not at all). Temperatures around 28–30°C fit all known species so far. In the lower limit most species show no apparent growth below 4–10°C (20°C in the case of *H. magadiensis* and *Z. palmae*), but *H. subglaciescola* is capable of growing at 0°C (Table 3).

## Preservation

A variety of media have been described for the routine maintenance of halomonads strains in

the laboratory. In many cases the same media served also for the isolation of the strains, whereas in others different formulations were employed. Some of these formulations, for instance the Artificial Organic Lake (AOL) medium of Franzmann et al. (1987), are intended to mimic the chemical composition of the environment from where the organisms are isolated. Once that isolation has been achieved many authors find it advantageous, especially if preparation of the isolation medium is too laborious, to employ a more general medium that permits the growth of both fresh isolates and reference strains. Thus, one of the most commonly employed media is MH, for moderately halophilic bacteria (Ventosa et al., 1982).

MH (Ventosa et al., 1982)

Yeast extract	10 g
Proteose peptone	5 g
Glucose	1 g
NaCl	81 g
MgCl <sub>2</sub> · 6H <sub>2</sub> O	7 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	9.6 g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.36 g
KCl	2 g
NaHCO <sub>3</sub>	0.06 g
NaBr	0.026 g

Dissolve ingredients and q.s. to 1 liter with distilled water. Adjust pH to 7.2 with 1 M KOH or NaOH. Add agar (20 g liter<sup>-1</sup>) for preparation of solid media. Adjust total saline content (commonly 10%) to any other desired value by lowering or raising proportionally the amounts of salts.

In a recent survey, Mata et al. (2002) employed MH medium at 7.5% salt content to maintain 104 *Halomonas* strains (including 21 type strains). For two of the strains, *H. magadiensis* NCIMB 13595<sup>T</sup> and *H. campisalis* ATCC 700597<sup>T</sup>, the pH was adjusted to 9.

For the species of the family Halomonadaceae with a lower salt requirement, commercial media such as Marine Agar (MA) can be satisfactorily employed (Yoon et al., 2001; Heyrman et al., 2002; Romanenko et al., 2002). Alternatively, other commercial media such as trypticase soy agar (TSA) can also be employed by adding NaCl (or a mixture of salts) or even no salts (for the nonhalophilic species).

Cultures on agar slants can be sealed and stored at 4–10°C. Although viability may last for much longer periods it is recommended that they be transferred regularly every 1–3 months.

For long-term preservation, lyophilization is advised. Prior to the vacuum drying, actively growing cells can be suspended on protecting fluids such as 5% inositol solution, and then the vials can be frozen by immersion into liquid nitrogen.

Cryopreservation at –80°C or under liquid nitrogen is also possible. To enhance survival of the cells they have to be suspended with a cryoprotectant such as 20% glycerol solution. Such prepared vials can also be stored at –20°C for middle-term preservation. However, since the quality of the culture may diminish faster (especially after frequent freeze-thawing) it is recommended that new stocks be prepared regularly.

## Identification

The members of the family Halomonadaceae cannot be defined by a reasonably large number of common-to-all features. The phenotypic heterogeneity of the family is also a handicap for the identification at the genus or species level unless a sufficient number of characters are determined. Recently, Mata et al. (2002) reviewed in depth the phenotypic features of the genus *Halomonas*, including the type strains of the 21 species regarded at that time to be within the genus and 83 additional strains corresponding to four of these species to assess the intraspecific variability. They reported a large number of traits not analyzed previously for all strains and found tests that are useful for distinguishing the species of the genus *Halomonas*. These included tests for physiological traits (facultatively anaerobic growth, poly-β-hydroxybutyric acid accumulation, and production of exopolysaccharides), biochemical traits (hydrolysis of starch and casein, tyrosine pigment, respiration on nitrite, lysine decarboxylase, lecithinase, phenylalanine deaminase, growth on MacConkey agar, and hemolysis), nutritional traits (growth on succinate, L-alanine and L-serine), and antimicrobial susceptibility (i.e., susceptibility to nitrofurantoin, rifampicin and trimethoprim-sulfamethoxazole), as well as utilization of four of the substrates included in the Biolog GN2 system (*N*-acetyl-D-galactosamine, *p*-hydroxyphenylacetic acid, sebacic acid, and D-serine).

Sequence comparison of rRNA genes (16S and 23S) has proved to be a valuable tool for the taxonomic classification of this group (Dobson et al., 1993; Mellado et al., 1995b; Dobson and Franzmann, 1996; Arahall et al., 2002a), and in the same way, it is very useful for identification purposes. However, researchers have to be aware of the limitation of this method, especially at the species level (see Prokaryote Characterization and Identification in Volume 1), and therefore other approaches should not be neglected.

Given the large pool of 16S rRNA (and to a lesser extent 23S rRNA) sequences of the group,

the possibility of designing oligonucleotide probes for the in situ identification of halomonads is great, but unfortunately it has not been addressed yet.

The chemotaxonomy of the group was reviewed by Franzmann and Tindall (1990). The 16 strains included in that study represent 12 of the current species of the genus *Halomonas* plus *Cobetia* (*Halomonas*) *marina* and *Chromohalobacter salexigens* (*Halomonas elongata* DSM 3043). For most species described thereafter, the fatty acid profile is available whereas the analysis of menaquinones or polar lipids has been addressed for only a few of them. However, for identification purposes these chemotaxonomic markers are of limited value at the species level. Moreover, since the results are affected by the methodology employed and the culture conditions of the strains, a full comparison of different studies is not feasible.

The Subcommittee on the Taxonomy of the Halomonadaceae has discussed the need for a publication on minimal standards for describing new genera and species of the Halomonadaceae, and a working team is currently discussing the features and methods to be included.

The phenotypic features and the G+C content of species of the genera *Halomonas* (Table 4), *Chromohalobacter* and *Cobetia* (Table 5) and the genera of the family Halomonadaceae (Table 6) are shown below.

## Physiology and Genetics

Osmoadaptation by some strains has been studied in more detail than any other physiological character. Osmoadaptation implies adaptation not only to a certain range of salinities but also to sudden changes in extracellular osmolarity. This is achieved thanks to a “salt-out” mechanism in which variable amounts of specific organic osmolytes are accumulated within the cytoplasm to balance the external osmotic pressure (Wohlfarth et al., 1990; Galinski, 1995; Kempf and Bremer, 1998; Vargas et al., 2004). The physiology of these (*Cobetia marina*, *H. alimentaria*, *H. campisalis*, *H. elongata*, *H. halodenitrificans*, *H. marisflavi*, *H. muralis* and *H. salina*) and other moderately halophilic bacteria has been extensively reviewed by Ventosa et al. (1998).

The species that exhibit a tolerance toward salts (which are the majority) usually depend upon a certain amount of NaCl in the environment for growth. For instance, *C. salexigens* requires at least 0.5 M NaCl in minimal medium (Cánovas et al., 1996). Recently, O'Connor and Csonka (2003) further characterized the ion requirements of *C. salexigens* and made the unexpected finding that while this organism

needs moderate concentrations of Na<sup>+</sup> and Cl<sup>-</sup> ions, its growth rate was stimulated by a number of other salts, indicating that *C. salexigens* requires a combination of NaCl and high ionic strength for optimal growth. However, *H. elongata* has a lower ionic requirement, and Cl<sup>-</sup> can be substituted by other anions like Br<sup>-</sup> or NO<sub>3</sub><sup>-</sup>, but not I<sup>-</sup> or SO<sub>4</sub><sup>-2</sup> (Vreeland and Martin, 1980a). *Halomonas halophila* grows on NaBr, Na<sub>2</sub>SO<sub>4</sub> and Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> as well as on NaCl but not on other sodium salts (Quesada et al., 1987). The role of K<sup>+</sup> in osmoregulation of *H. elongata* was investigated by Kraegeloh and Kunte (2002). At lower salinities (0.51 M NaCl), K<sup>+</sup> was the main cytoplasmic solute. At a double NaCl concentration, the same K<sup>+</sup> content was measured, but this value was surpassed by the content of ectoine, the predominant cytoplasmic solute at higher salinity. Cells upon osmotic upshock were found to accumulate simultaneously ectoine and potassium glutamate, reaching levels greater than those found in cells adapted to high salinity (not stressed). The increase in K<sup>+</sup> was long-lasting (more than 120 min) and not transient.

*Chromohalobacter salexigens* DSM 3043<sup>T</sup> (previously *Halomonas elongata* DSM 3043; Arahal et al., 2001b) and *H. elongata* DSM 2581<sup>T</sup> have been used as model organisms for a large number of osmoadaptation studies in moderate halophiles that have been reviewed recently by Ventosa et al. (1998), Kunte (2004) and Vargas et al. (2004).

Many of the osmoadaptation mechanisms of *C. salexigens* and *H. elongata* are now understood at the molecular level (Kunte, 2004; Vargas et al., 2004). When present in the extracellular environment, *C. salexigens* is able to take up ectoine, hydroxyectoine, betaine, choline, and choline-O-sulfate. At least three transport systems have been inferred. Into the cell, choline is oxidized to betaine and the genes involved—*betIBA* genes—have been cloned and characterized (Cánovas et al., 1998b; Cánovas et al., 2000). In *H. elongata*, Grammann et al. (2002) have described the isolation and molecular characterization of an osmoregulated transport system—TeaABC—showing high affinity for ectoine and hydroxyectoine.

When grown in a minimal medium, *C. salexigens* synthesizes de novo the compatible solutes ectoine and its derivative hydroxyectoine. The sequences of the genes involved in the synthesis of ectoine—*ectABC*—were isolated and characterized (Cánovas et al., 1998a). They code for a diaminobutyric acid acetyltransferase, a diaminobutyric acid aminotransaminase, and an ectoine synthase, respectively (Table 7). The *C. salexigens* EctA, EctB and EctC genes showed the same organization as well as a significant degree of sequence identity to the genes of

Table 4. Phenotypic characteristics and G+C content of DNA of species of the genus *Halomonas*.<sup>a,b</sup>

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Poly-β-alkanoates	nd	+	+	+	+	+	+	+	nd	+	-	+	+	+	+	+	nd	+	+	+	+	+	+
Exopolysaccharide production	nd	-	v	-	-	-	+	-	nd	-	-	-	-	-	-	-	nd	-	-	+	-	-	-
Motility	nd	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	v	+	+	+
Facultative anaerobe	nd	-	-	-	-	+	-	-	nd	-	-	-	-	-	-	-	nd	+	-	-	-	-	-
Oxidase	+	+	+	-	+	v	-	+	+	+	v	+	+	+	+	+	+	+	+	+	+	+	+
Acid from																							
Adonitol	nd	+	-	+	-	v	-	+	nd	-	-	-	-	+	-	+	nd	-	+	-	-	-	-
L-Arabinose	nd	+	-	+	-	+	-	+	+	-	v	v	-	+	-	+	nd	-	+	-	-	-	-
D-Fructose	nd	+	-	+	-	v	-	+	nd	-	+	+	-	+	-	+	nd	-	+	-	-	-	-
D-Galactose	nd	+	-	+	-	v	-	+	-	-	+	+	-	+	-	+	nd	v	+	-	v	-	-
D-Glucose	-	+	-	+	-	+	-	+	+	-	+	+	-	+	-	+	nd	+	+	-	v	-	v
myo-Inositol	nd	+	-	+	-	-	-	+	nd	-	-	v	-	-	-	+	nd	-	+	-	v	-	-
Lactose	nd	+	-	+	-	v	-	v	+	-	v	-	-	+	-	-	nd	-	-	-	v	-	-
Maltose	nd	+	-	+	-	+	-	+	nd	-	+	+	-	+	-	+	nd	-	+	-	v	-	-
D-Mannitol	nd	+	-	+	-	v	-	+	-	-	v	v	-	+	-	+	nd	-	+	-	-	-	v
D-Mannose	nd	+	-	+	-	v	-	+	-	-	v	+	-	+	-	+	nd	v	-	-	-	-	-
D-Melezitose	nd	+	-	+	-	v	-	+	nd	-	v	+	-	+	-	+	nd	-	+	-	-	-	-
L-Rhamnose	nd	+	-	+	-	-	-	+	nd	-	-	+	-	+	-	+	nd	-	+	-	-	-	-
Sucrose	nd	+	-	+	-	+	-	+	+	-	+	v	-	+	-	+	nd	-	+	-	-	-	-
D-Salicin	nd	+	-	+	-	+	-	+	nd	-	v	+	-	+	-	+	nd	-	+	-	-	-	-
D-Sorbitol	nd	+	-	+	-	v	-	+	nd	-	-	+	-	+	-	+	nd	-	+	-	-	-	-
Sorbitose	nd	+	-	+	-	-	-	+	nd	-	-	-	-	+	-	+	nd	-	+	-	-	-	-
D-Trehalose	nd	+	-	+	-	+	-	+	nd	-	-	v	-	+	-	+	nd	-	+	-	-	-	-
Hydrolysis of																							
Starch	nd	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	nd	-	-	-	-	-	-
Esculin	-	-	-	+	-	v	+	v	-	-	+	+	-	+	-	-	+	-	+	-	-	+	-
Gelatin	-	-	-	v	-	v	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Casein	-	-	-	v	-	v	-	-	-	-	+	-	-	-	-	-	-	-	-	-	v	+	-
Tween 20	nd	+	+	+	+	v	-	+	nd	-	-	-	+	+	+	+	nd	-	+	+	+	+	-
Tween 80	-	v	-	-	v	-	+	v	+	-	+	-	v	-	-	v	nd	-	+	+	-	-	-
DNA	nd	v	+	v	+	v	v	+	nd	-	+	+	+	+	-	-	nd	nd	+	+	v	v	v
Tyrosine	-	-	-	-	+	-	v	-	nd	+	-	-	+	-	-	-	-	nd	v	-	-	-	-

(Continued)

Table 4. Continued

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Tyrosine pigment	nd	-	-	-	-	-	+	-	nd	-	-	+	-	-	+	-	nd	-	-	-	-	-	-
Lysine decarboxylase	nd	-	-	-	-	+	-	v	nd	v	+	-	-	-	-	-	nd	-	-	-	-	-	-
Ornithine decarboxylase	nd	-	-	-	-	+	-	+	nd	v	+	v	-	-	-	+	nd	-	-	-	-	-	-
H <sub>2</sub> S production	nd	-	-	-	+	v	+	-	nd	v	+	+	+	-	+	+	+	-	+	+	+	v	+
Nitrate reduction	+	+	+	+	+	+	-	-	-	+	v	+	+	-	+	-	nd	-	-	-	-	-	-
Nitrite reduction	nd	+	+	+	+	v	-	v	nd	v	+	v	-	+	+	-	nd	-	-	-	-	-	-
Phosphatase	nd	v	v	v	+	+	+	v	nd	v	+	v	+	+	v	-	nd	-	-	-	v	-	-
Urease	+	-	-	-	v	+	+	+	-	-	v	v	+	-	-	+	-	+	v	+	v	+	+
Lecthinase	nd	-	-	-	v	-	-	-	nd	-	-	v	-	+	-	-	nd	-	-	-	-	-	v
Phenylalanine deaminase	nd	-	-	-	v	-	-	-	nd	-	-	v	-	+	-	-	nd	-	-	-	-	-	-
ONPG	nd	-	-	-	v	+	+	v	nd	-	-	-	-	-	-	-	nd	-	-	-	-	-	-
O/F (D-glucose)	nd	O	O	F	O	v	O	O	nd	O	O	F	O	O	O	F	nd	O	v	O	O	O	O
Gluconate oxidation	nd	+	+	+	+	+	v	-	nd	+	+	-	+	+	+	+	nd	+	+	+	+	+	+
Growth on MacConkey	nd	+	+	+	+	+	+	+	nd	+	+	+	+	+	+	+	nd	+	+	+	+	+	+
Growth on cetrimide agar	nd	-	-	-	+	+	+	-	nd	-	+	-	+	-	+	-	nd	-	-	-	-	-	-
Respiration on nitrate	nd	-	+	+	+	v	-	v	nd	+	-	-	-	-	+	-	nd	-	-	v	-	-	-
Respiration on nitrite	nd	-	+	+	+	-	-	-	nd	+	-	-	-	-	-	-	nd	-	-	-	-	-	-
Hemolysis	nd	-	-	-	-	-	-	-	nd	-	-	-	-	+	-	-	nd	-	-	-	-	-	-
Growth on																							
Esculin	nd	-	-	-	-	v	-	v	nd	+	+	+	+	-	-	v	nd	-	-	-	-	-	-
L-Arabinose	nd	v	-	+	+	v	+	v	nd	-	v	+	+	v	+	v	+	-	v	+	v	v	-
D-Cellobiose	nd	-	+	v	-	+	+	v	nd	-	+	v	-	+	-	v	+	-	-	v	-	v	v
D-Fructose	nd	v	+	+	+	+	v	v	nd	+	+	+	+	+	+	v	+	-	+	v	v	v	v
D-Galactose	nd	-	-	v	v	+	+	v	nd	+	+	+	+	+	+	+	+	-	+	v	v	v	v
D-Glucose	nd	+	+	+	v	+	v	v	nd	+	+	v	v	+	v	v	+	-	+	v	v	v	v
Lactose	nd	v	-	v	+	+	+	v	nd	-	+	-	v	+	-	v	+	-	-	-	-	-	-
Maltose	nd	-	+	+	+	v	+	+	nd	-	-	+	-	+	+	+	nd	v	+	v	v	v	v
D-Mannose	nd	-	-	+	+	+	+	+	nd	+	+	+	+	+	+	+	nd	v	+	v	v	v	v
D-Melezitose	nd	+	-	-	+	+	+	+	nd	+	+	+	+	+	+	+	nd	-	+	+	+	+	+
D-Raffinose	nd	-	-	-	-	v	+	v	nd	-	-	v	+	-	-	v	+	-	+	+	+	+	+
L-Rhamnose	nd	-	-	v	-	+	+	-	nd	-	-	v	v	-	v	-	nd	v	-	v	-	-	-
Ribose	nd	-	-	+	v	+	+	-	nd	+	+	-	v	+	+	v	nd	v	-	v	-	-	v





Table 5. Phenotypic characteristics and G+C content of DNA of species of the genera *Chromohalobacter* and *Cobetia*.<sup>a</sup>

Characteristic	<i>C. canadensis</i>	<i>C. israelensis</i>	<i>C. marismortui</i>	<i>C. salexigens</i>	<i>Cobetia marina</i>
Acid from					
L-Arabinose	nd	nd	+	+	-
Lactose	+	+	+	v	-
Maltose	-	+	+	+	-
Sucrose	-	+	+	+	v
D-Trehalose	-	-	+	-	v
D-Xylose	nd	nd	+	+	-
Indole	+	+	-	-	-
Citrate	-	-	+	+	+
Lysine decarboxylase	+	+	-	v	-
Ornithine decarboxylase	+	+	-	v	-
NO <sub>3</sub> <sup>-</sup> reduction	+	+	-	v	-
Phosphatase	+	-	-	nd	+
Urease	-	-	-	+	+
ONPG	nd	nd	-	v	+
O/F (D-glucose)	nd	nd	O	F	O
Growth on	nd	nd	Nd	nd	nd
Esculin	+	+	-	+	+
L-Arabinose	+	nd	Nd	v	-
D-Cellobiose	+	+	-	v	v
D-Fructose	+	nd	-	+	+
Maltose	-	nd	+	+	v
L-Rhamnose	nd	nd	+	-	-
Ribose	-	nd	+	+	+
Sucrose	+	+	+	+	-
Starch	v	+	+	+	-
D-Xylose	+	nd	+	+	-
Citrate	+	nd	-	+	v
Fumarate	nd	nd	-	+	+
Malonate	nd	nd	-	+	v
Propionate	-	nd	-	+	+
Succinate	+	nd	-	+	+
Adonitol	nd	nd	+	-	-
Sorbitol	-	nd	+	v	-
L-Lysine	nd	nd	-	+	-
G+C content (mol%)	62	65	62.3	64.2	63

Symbols and abbreviations: +, positive; -, negative; v, variable between different studies; nd, not determined; ONPG, 2-nitrophenyl-β-D-galactopyranoside; O, oxidative; and F, fermentative.

<sup>a</sup>All species are rod-shaped, catalase positive and utilize gluconate. None have phenylalanine deaminase or hydrolyze esculin, gelatin and starch.

Data from references in Table 1.

ectoine synthesis in *H. elongata* (Göller et al., 1998), *Marinococcus halophilus* (Louis and Galinski, 1997) and *Sporosarcina pasteurii* (Kuhlmann and Bremer, 2002). Thus, it seems that the ectoine biosynthetic route, which was first elucidated at the biochemical level in the closely related *H. elongata* (Peters et al., 1990; Ono et al., 1999), is evolutionarily well conserved in all ectoine-producing bacteria characterized so far. Very recently, Calderón et al. (2004) investigated the long-term regulation of ectoine synthesis in *C. salexigens*, with emphasis in the transcriptional regulation, and found preliminary indications of great complexity of this process. S1 protection analyses performed with RNA extracted from cells grown in minimal medium at low (0.75 M NaCl) or high (2.5 M NaCl) osmolarity suggested

the existence of four promoters upstream of *ectA*. Two of these (*PectA1* and *PectA2*) might be recognized by the main vegetative sigma factor  $\sigma^{70}$ , and one (*PectA3*) might be dependent on the general stress factor  $\sigma^S$ . The S1 protection assays suggested that *PectA1* and *PectA3* may be osmoregulated promoters. In addition, an internal promoter showing sequences homologous to promoters dependent on the heat-shock sigma factor  $\sigma^{32}$  was found upstream of *ectB*. Transcription from *PectA* in *C. salexigens* followed a pattern typical of  $\sigma^S$ -dependent promoters, and was reduced by 50% in an *Escherichia coli rpoS* background. These data strongly suggest the involvement of the general stress factor  $\sigma^S$  in *ectABC* transcription in *C. salexigens*. Expression of *PectA-lacZ* and *PectB-lacZ* transcriptional

Table 6. Phenotypic characteristics and G+C content of DNA of the genera of Halomonadaceae.

Characteristic	<i>Halomonas</i>	<i>Chromohalobacter</i>	<i>Cobetia</i>	<i>Carnimonas</i>	<i>Zymobacter</i>
Oxidase	+	–	–	+	–
Acid from					
L-Arabinose	D	nd	–	–	nd
D-Fructose	D	nd	+	+	nd
D-Glucose	D	nd	+	+	nd
Lactose	D	+	–	nd	nd
Maltose	D	+	–	+	nd
D-Mannitol	D	nd	+	+	nd
D-Mannose	D	+	v	+	nd
D-Melezitose	D	nd	–	–	nd
Sucrose	D	+	v	nd	nd
Hydrolysis of					
Starch	–	–	–	+	–
Esculin	D	–	–	+	nd
Casein	–	–	v	–	nd
DNA	D	–	+	–	nd
Indole production	–	D	–	–	–
Methyl red	nd	D	–	nd	+
Voges-Proskauer	nd	–	nd	–	+
Citrate utilization	nd	D	+	nd	–
Arginine dihydrolase	nd	nd	–	–	–
Lysine decarboxylase	–	D	–	nd	–
Ornithine decarboxylase	D	D	–	nd	–
H <sub>2</sub> S production	D	–	v	nd	nd
Nitrate reduction	D	+	–	–	–
Phosphatase	D	D	+	nd	nd
Urease	D	–	+	–	nd
Lecithinase	D	nd	v	–	nd
Phenylalanine deaminase	–	–	–	+	–
ONPG	–	nd	+	+	–
O/F (D-glucose)	D	nd	O	nd	F
Growth on MacConkey	+	nd	+	+	nd
Growth on cetrimide agar	D	nd	+	+	nd
Growth on					
Esculin	D	+	+	nd	nd
L-Arabinose	D	nd	–	–	nd
D-Fructose	D	D	+	nd	nd
D-Glucose	D	+	+	nd	nd
Lactose	D	+	v	nd	nd
Maltose	D	D	v	+	nd
D-Mannose	D	+	v	+	nd
Ribose	D	D	+	nd	nd
Sucrose	D	+	–	nd	nd
Starch	D	+	–	nd	nd
D-Xylose	D	+	–	nd	nd
Citrate	D	D	v	+	nd
Gluconate	D	+	+	+	nd
Propionate	D	D	+	nd	nd
Succinate	+	D	+	nd	nd
Glycerol	+	+	+	nd	nd
<i>myo</i> -Inositol	D	D	+	nd	nd
D-Mannitol	D	+	+	–	nd
Sorbitol	D	D	–	nd	nd
G+C content (mol%)	52–68	62–66	63	56	56

Symbols and abbreviations: +, positive; –, negative; v, variable between different studies; D, different results for the species included in the genus; nd, not determined; ONPG, 2-nitrophenyl-β-D-galactopyranoside; O, oxidative; and F, fermentative.

fusions was very high at low salinity, suggesting that *ectABC* may be a partially constitutive system. Both transcriptional fusions were induced during continuous growth at high temperature

and reduced in cells grown in the presence of osmoprotectants (ectoine or glycine betaine) or the DNA gyrase inhibitor nalidixic acid. Moreover, *PectA-lacZ* expression was negatively mod-

Table 7. Genes sequenced and characterized from members of the family Halomonadaceae.

Strain	Gene (gene product)	Accession <sup>a</sup>	References
<i>C. marina</i> N-1	<i>alg</i> (alginate lyase)	AB018795 (1896bp)	J. Kraiwattanapong, T. Ooi and S. Kinoshita, unpublished
<i>C. salaxigens</i> DSM 3043 <sup>T</sup>	<i>betIBA</i> (regulatory protein, betaine aldehyde dehydrogenase, and choline dehydrogenase)	AJ238780 (4597bp)	Cánovas et al., 2000
<i>C. salaxigens</i> DSM 3043 <sup>T</sup>	<i>ectABC</i> (diaminobutyric acid acetyltransferase, L-diaminobutyric acid aminotransferase, and ectoine synthase)	AJ011103 (2869bp)	Cánovas et al., 1998a
<i>H. elongata</i> DSM 2581 <sup>T</sup>	<i>ectABC</i> (diaminobutyric acid acetyltransferase, L-diaminobutyric acid aminotransferase, and ectoine synthase)	AF031489 (2744bp)	Goller et al., 1998
<i>H. elongata</i> DSM 2581 <sup>T</sup>	<i>teaABC</i> (periplasmic substrate-binding protein, and transmembrane proteins, small and large)	a061646 (3993bp)	Grammann et al., 2002
<i>H. elongata</i> DSM 2581 <sup>T</sup>	<i>trkAH</i> (NAD binding protein, and transmembrane protein)	a437838 (2866bp)	A. Kraegeloh, B. Amendt and H.-J. Kunte, unpublished
<i>H. elongata</i> DSM 2581 <sup>T</sup>	<i>trkI</i> (transmembrane protein)	AY437839 (1479bp)	A. Kraegeloh, B. Amendt and H.-J. Kunte, unpublished
<i>H. eurihalina</i> F2-7	<i>carAB</i> (carbamoylphosphate synthetase, small and large subunits)	AJ431666 (4965bp)	Llamas et al., 2003
<i>H. halodenitrificans</i> IFO 14912 <sup>T</sup>	<i>narK<sub>2</sub>LXKGH</i> (nitrate transporter, nitrate extrusion protein, nitrate or nitrite response regulator, nitrate or nitrite sensor protein, nitrate transporter, and nitrate reductase [alpha and beta subunits])	AB076402 (14936bp)	A. Asada, T. Sakurai, A. Moriyama and N. Sakurai, unpublished
<i>H. halodenitrificans</i> IFO 14912 <sup>T</sup>	<i>norCBQ</i> (nitric oxide reductase, cytochrome <i>c</i> subunit, cytochrome <i>b</i> subunit and NorQ)	AB010889 (2918bp)	Sakurai and Sakurai, 1998
<i>H. meridiana</i> DSM 5425 <sup>T</sup>	<i>amyH</i> ( $\alpha$ -amylase)	AJ239061 (1600bp)	Coronado et al., 2000b
<i>H. salina</i> AS11	<i>aldh</i> (aldehyde dehydrogenase)	AF284553 (1680bp)	Sripo et al., 2002
<i>Halomonas</i> sp. #593	<i>ndk</i> (nucleoside diphosphate kinase)	AB085190 (9151bp)	Yonezawa et al., 2003
<i>Halomonas</i> sp. SK1	<i>hktA</i> (catalase)	AB069960 (2658bp)	Phuchatoen and Shinozawa, 2002
<i>Z. palmae</i> ATCC 51623 <sup>T</sup>	<i>pdc</i> (pyruvate decarboxylase)	AF474145 (2007bp)	Raj et al., 2002

Abbreviation: <sup>T</sup>, type strain.<sup>a</sup>Lengths (in parenthesis) correspond to those of the deposited sequences and thus they might not correspond to the length of the gene.

Table 8. Predicted genome sizes of members of the family Halomonadaceae.

Strain	Genome size <sup>a</sup> (Mb)	Restriction enzyme	References
<i>C. israelensis</i> ATCC 43985 <sup>T</sup>	2.5	<i>SpeI</i>	Mellado et al., 1998
<i>C. marismortui</i> ATCC 17056 <sup>T</sup>	2.2/2.3	<i>SpeI/SwaI</i>	Mellado et al., 1998
<i>C. marismortui</i> A-100	2.0/2.3	<i>SpeI/SwaI</i>	Mellado et al., 1998
<i>C. marismortui</i> A-492	1.8/2.0	<i>SpeI/SwaI</i>	Mellado et al., 1998
<i>C. salexigens</i> ATCC 33174	2.4/2.8	<i>SpeI/SwaI</i>	Mellado et al., 1998
<i>H. elongata</i> ATCC 33173 <sup>T</sup>	2.2/2.8	<i>SpeI/SwaI</i>	Mellado et al., 1998
<i>H. eurihalina</i> ATCC 49336 <sup>T</sup>	2.2/2.2	<i>SpeI/SwaI</i>	Mellado et al., 1998
<i>H. eurihalina</i> F2-12	2.3/2.3	<i>SpeI/SwaI</i>	Mellado et al., 1998
<i>H. eurihalina</i> F2-7	2.5	<i>SwaI</i>	Llamas et al., 2002
<i>H. halmophila</i> ATCC 19717 <sup>T</sup>	1.5	<i>SpeI</i>	Mellado et al., 1998
<i>H. halodurans</i> ATCC 29686 <sup>T</sup>	1.8/1.9	<i>SpeI/SwaI</i>	Mellado et al., 1998
<i>H. subglaciescola</i> UQM 2927 <sup>T</sup>	2.5/2.5	<i>SpeI/SwaI</i>	Mellado et al., 1998

Abbreviation: <sup>T</sup>, type strain.

<sup>a</sup>Calculated according to the fragments obtained after restriction with the enzymes shown in next column.

ulated in cells grown with an excess of iron (FeCl<sub>3</sub>). Table 7 shows other genes from members of the family Halomonadaceae that have been sequenced and characterized.

Estimations of the genome size of eleven *Halomonas* and *Chromohalobacter* strains by using pulsed-field gel electrophoresis are in the range 1.5–2.8 Mb (Mellado et al., 1998). The genome size of *H. eurihalina* F2-7 has been determined recently as 2.5 Mb, together with its topology and a preliminary physical map (Llamas et al., 2002). The type strain of *H. maura* has a larger chromosome (3.5 Mb; Quesada et al., 2004). A summary of estimations of the genome size of some halomonads is presented in Table 8.

Although research on the nonhalophilic species of the family Halomonadaceae is less abundant, some studies have been conducted. Thus, the pyruvate decarboxylase gene, *pdh*, of *Z. palmarum* has been cloned and characterized (Raj et al., 2002). Its product is the key enzyme in all homo-ethanol fermentations, and so far it has only been identified in three other bacteria. Among eukaryotes, it is absent in animals but widely distributed in plants, yeasts and fungi.

The presence of plasmids (and megaplasmids) in strains of *Halomonas*, *Chromohalobacter* and *Cobetia* has been investigated (Table 9). Plasmid pHE1 (from *C. salexigens* ATCC 33174) was selected for its small size (4.2 kb) and served for the construction of a shuttle vector, pHS15, for related moderately halophilic bacteria (Vargas et al., 1995). The mobilization (*mob*) region of plasmid pHE1 has been characterized revealing the presence of four open reading frames (Vargas et al., 1999). In the same way, plasmids pEE3 and pEE5 are derivatives of plasmid pCM1 from *C. marismortui* (Mellado et al., 1995c). More recently, Argandoña et al. (2003) have discovered that many Halomonadaceae strains

harbor large extrachromosomal DNA elements, although they are technically very difficult to identify by using standard protocols. Under appropriate culture conditions and applying a modified gel electrophoresis method, they found that almost all species studied harbor two plasmids of about 70 kb and 600 kb, and some species carry other smaller extrachromosomal DNA elements (Table 9).

## Ecology

It can be inferred that halomonads are very ubiquitous and versatile chemoheterotrophic microorganisms from the variety of habitats they are found in (Table 2) and from their phenotypic heterogeneity (Mata et al., 2002). Studies on the ecology of hypersaline habitats are of particular interest because of their reduced diversity with increasing salinity. When the community of moderately halophilic bacteria is analyzed by numerical taxonomy approaches in salt lakes and brines, halomonads (or halomonad-like) strains are commonly isolated (Ventosa et al., 1998). However, such classical studies have been lately surpassed by molecular techniques or a combination of both.

Information derived from publicly available 16S rRNA sequences reveals that both isolates and environmental clones are being isolated from a wide range of environments, such as continental shelf sediments collected off Antarctica, deep-sea sediment from Pacific Ocean, Great Salt Plains of Oklahoma, Lake Texcoco (Mexico), the Hawaiian Archipelago, tropical ecosystems, or the Arctic Sea, to name a few. They are present in bulk water, oxic and anoxic transition zones, and anoxic basins or sediments at almost any range of salinities and a wide range of pH and temperature.

Table 9. Plasmids (and megaplasmids) from Halomonadaceae strains.

Strain	Plasmids	Sizes (kb)	References
<i>C. marina</i> ATCC 25374 <sup>T</sup>	Unnamed	601, 71.2, 5.4	Argandoña et al., 2003
<i>C. canadensis</i> ATCC 43984 <sup>T</sup>	Unnamed	620, 70.2	Argandoña et al., 2003
<i>C. israelensis</i> ATCC 43985 <sup>T</sup>	pHI1	48	Vargas et al., 1995
<i>C. israelensis</i> CECT 5287 <sup>T</sup>	Unnamed	48	Argandoña et al., 2003
<i>C. marismortui</i> ATCC 17056 <sup>T</sup>	pCM1	17.5	Mellado et al., 1995a
<i>C. marismortui</i> DSM 6770 <sup>T</sup>	Unnamed	592, 119.3, 67.5	Argandoña et al., 2003
<i>C. salexigens</i> ATCC 33174	pHE1	4.2	Vargas et al., 1995
<i>H. aquamarina</i> DSM 30161 <sup>T</sup>	Unnamed	600, 74	Argandoña et al., 2003
<i>H. desiderata</i> DSM 9502 <sup>T</sup>	Unnamed	595, 88.5, 25.8	Argandoña et al., 2003
<i>H. elongata</i> ATCC 33173 <sup>T</sup>	pMH1	11.5	Fernández-Castillo et al., 1992
<i>H. elongata</i> CECT 4279 <sup>T</sup>	Unnamed	595, 70.5	Argandoña et al., 2003
<i>H. eurihalina</i> ATCC 49336 <sup>T</sup>	Unnamed	610, 8.1, 5.8	Argandoña et al., 2003
<i>H. eurihalina</i> F2-7	pVE1, pVE2	8.1, 5.8	Llamas et al., 1997
<i>H. eurihalina</i> H-1	pVE3	5.3	Llamas et al., 2002
<i>H. eurihalina</i> H-217	pVE4	16	Llamas et al., 2002
<i>H. eurihalina</i> H-236	pVE5	6.5	Llamas et al., 2002
<i>H. eurihalina</i> X8	Unnamed	610	Argandoña et al., 2003
<i>H. halmophila</i> ATCC 19717 <sup>T</sup>	pMH1	11.5	Fernández-Castillo et al., 1992
<i>H. halmophila</i> ATCC 19717 <sup>T</sup>	Unnamed	595, 73.8	Argandoña et al., 2003
<i>H. halodenitrificans</i> CECT 5012 <sup>T</sup>	Unnamed	600	Argandoña et al., 2003
<i>H. halodurans</i> LGM 10144 <sup>T</sup>	Unnamed	601; 75; 5.4	Argandoña et al., 2003
<i>H. halophila</i> CCM 3662 <sup>T</sup>	pMH1	11.5	Fernández-Castillo et al., 1992
<i>H. halophila</i> CCM 3662 <sup>T</sup>	Unnamed	595, 73.8	Argandoña et al., 2003
<i>H. magadii</i> NCIMB 13595 <sup>T</sup>	Unnamed	602, 68, 7.6, 5.7	Argandoña et al., 2003
<i>H. maura</i> CECT 5298 <sup>T</sup>	Unnamed	619, 70.7	Argandoña et al., 2003
<i>H. maura</i> M15	Unnamed	620, 70	Argandoña et al., 2003
<i>H. meridiana</i> DSM 5425 <sup>T</sup>	Unnamed	597, 79.2	Argandoña et al., 2003
<i>H. pantelleriensis</i> DSM 9661 <sup>T</sup>	Unnamed	595, 73.4	Argandoña et al., 2003
<i>H. salina</i> CECT 5288 <sup>T</sup>	Unnamed	601, 71.2	Argandoña et al., 2003
<i>H. subglaciescola</i> DSM 4683 <sup>T</sup>	Unnamed	620, 68	Argandoña et al., 2003
<i>H. subglaciescola</i> UQM 2927 <sup>T</sup>	pHS1	ca. 70	Vargas et al., 1995
<i>H. variabilis</i> DSM 3051 <sup>T</sup>	Unnamed	604.8	Argandoña et al., 2003
<i>H. venusta</i> ATCC 27125 <sup>T</sup>	Unnamed	592, 144.4, 75	Argandoña et al., 2003

Abbreviation: <sup>T</sup>, type strain.

Table 10. Organic compounds and chemicals that can be degraded or transformed by Halomonadaceae strains under hypersaline conditions.

Compound	Organism	NaCl (% w/v)	References
Aminomethane sulfonate	<i>C. marismortui</i> VH1	5–15	Ternan and McMullan, 2002
Organophosphonates	<i>C. marismortui</i> VH1	5–15	Hayes et al., 2000
Benzoate	<i>H. halodurans</i>	3–20	Rosenberg, 1983
Formaldehyde	<i>Halomonas</i> sp. MA-C	0–20	Azachi et al., 1995
Phenol	<i>H. campisalis</i> A4 <sup>T</sup>	0–15 <sup>a</sup>	Alva and Peyton, 2003
Phenol	<i>H. venusta</i>	8	Muñoz et al., 2001
Phenol	<i>Halomonas</i> sp.	1–14	Hinteregger and Streichsbier, 1997
Selenate	<i>Halomonas</i> sp. MPD-51 (and three more isolates)	0–32.5	de Souza et al., 2001
Uranium compounds	<i>Halomonas</i> sp. WIPP1A	20	Francis et al., 2000

Abbreviation: <sup>T</sup>, type strain.

<sup>a</sup>pH 8–11.

Most probable number (MPN) estimates of halotolerant microorganisms in hydrothermal-vent and pelagic waters of the North and South Pacific showed that up to 28% of the total microbial community was halotolerant. Fourteen isolates from these MPN enrichments

were identified by comparison of partial 16S rRNA gene sequences, and eight were found to belong to the genus *Halomonas* (Kaye et al., 2000).

Halomonads have also been found during the characterization of microbial assemblages asso-

ciated with high-temperature petroleum reservoirs (Orphan et al., 2000). Indeed, they represented the main group (9.4%) of a clone library generated from total community DNA collected from production wellheads (mean depth 1700 m below the sea floor) in California using universally conserved 16S rRNA-targeted polymerase chain reaction primers. The closest relatives to the halomonads phylotypes were *H. variabilis* (20 clones, 16S rRNA similarity range 98.5–99.6%) and *H. pacifica* (one clone, 16S rRNA similarity 97.2%).

A recent study has been conducted on the microbial communities from a subsea floor sediment core from the southwestern Sea of Okhotsk by cultivation-independent and cultivation-dependent analyses (Inagaki et al., 2003). Of 322 bacterial 16S rRNA clones, 264 (82.0%) grouped with Gammaproteobacteria, and among them, the predominant groups belonged to the genera *Halomonas*, *Methylophaga* and *Psychrobacter*. The *Halomonas* relatives included 147 clones (45.7%), of which two large groups were closely related to *H. variabilis* and *H. meridiana*. With respect to the isolates (181 strains selected on the basis of colony morphology, sample depth and cultivation conditions), the Gammaproteobacteria were again the main group (93.3% of isolates). Moreover, the most predominant colony phylotype accounted for 82 isolates (45.3%) and exhibited around 98% 16S rRNA sequence similarity with *H. variabilis*.

### Disease

Members of the Halomonadaceae are not pathogenic. There is one case report on the isolation of *H. venusta* from a human infection in a wound that originated from a fish bite (Von Graevenitz et al., 2000); however the identification of the organism alone does not prove its pathogenicity.

### Applications

The species of the Halomonadaceae can be used for several biotechnological purposes and, as in the case of other extremophilic microorganisms, many different applications have been suggested. The biotechnological potential and applications of moderately and halotolerant microorganisms have been reviewed in detail recently (Ventosa et al., 1998; Margesin and Schinner, 2001; Mellado and Ventosa, 2003). Some of the most promising applications of members of Halomonadaceae include the production of compatible solutes as well as extracellular compounds such as exopolysaccharides and enzymes, and their use in environmental bioremediation processes.

Compatible solutes are known for their stabilizing and protective effect on enzymes, nucleic acids, cell structures or whole cells subjected to low water activities, temperature stress, and other adverse conditions (Lippert and Galinski, 1992; Galinski, 1993; Knapp et al., 1999). A “bacterial milking” process to obtain ectoine from *Halomonas elongata* has been developed and patented (Sauer and Galinski, 1998) and later industrially exploited by Bitop (Witten, Germany). The process is based on subjecting the bacteria repeatedly to osmotic shocks. An osmotic down-shock permits the excretion of the intracellular ectoine to the surrounding medium while subsequent exposure of the cells to a hyperosmotic shock quickly restores the original level of ectoine (Sauer and Galinski, 1998). Ectoine and its derivative hydroxyectoine are used in the cosmetic industry because of their moisturizing properties. The potential use of the *C. salexigens* and *H. elongata* genes (responsible for the synthesis of ectoine) to obtain agriculturally important transgenic organisms tolerant to osmotic stress has already been proposed (Vargas et al., 2004). Nakayama et al. (2000) obtained transgenic cultured tobacco cells that accumulated the compatible solute ectoine from *H. elongata*, exhibiting a normal growth pattern under hyperosmotic conditions.

Strains from several species of *Halomonas* are of interest as producers of exopolysaccharides (EPS). These include *H. maura*, *H. eurihalina*, *H. salina* and *H. halophila* (Quesada et al., 2004), as well as other recent isolates that may represent new *Halomonas* species (E. Quesada et al., personal communication). The nutritional and environmental factors influencing the production of EPS have been thoroughly investigated by Quesada and coworkers (Quesada et al., 1993; Béjar et al., 1996; Béjar et al., 1998; Bouchotroch et al., 1999; Bouchotroch et al., 2000; Martínez-Checa et al., 2002). Yield can reach 1–3 g of EPS per liter of medium after five days of cultivation. Glucose and sucrose are the most efficient carbon sources (Béjar et al., 1996) although many other nutrients can be used including end products such as molasses from sugar beet (Quesada et al., 2004). Production of the EPS is not inhibited by the presence of crude oil. Moreover, the composition of the biopolymer is different under such conditions showing a highly efficient emulsifying activity towards crude oil (Calvo et al., 2002). Interestingly, it has been observed that strains of *H. maura* and *H. eurihalina* produce more EPS at salt concentrations below 7.5% (w/v), which are suboptimal for growth (Quesada et al., 1993; Bouchotroch et al., 2000). As for the chemical composition of the EPSs produced from *Halomonas* strains, they are anionic poly-

mers composed mainly of carbohydrates and a minor fraction of proteins, uronic acids and acetyls. Sulfate substituents have also been detected (in some strains exceeding 20% of the dry weight), and together with the uronic acids, make the EPS anionic. Depending on their composition EPSs have different functional properties and thus different applications, which include immunomodulation, biotransformation of heavy-metal polluted environments, crude oil emulsification, viscosity enhancement in foods, among others (Quesada et al., 2004). The properties of some of these EPSs such as maura, H28 or V2-7 have been studied in detail highlighting in each case their biotechnological potentials (Pérez-Fernández et al., 2000; Martínez-Checa et al., 2002; Arias et al., 2003).

Another interesting field is the production of extracellular enzymes (i.e., amylases, proteases and nucleases) by moderately halophilic representatives of the family. Although few studies have been carried out, this is a promising subject that has been reviewed very recently (Mellado et al., 2004). Sánchez-Porro et al. (2003) studied the diversity of moderately halophilic bacteria able to produce several extracellular hydrolytic enzymes (amylases, DNases, lipases, proteases and pullulanases) by screening different hypersaline locations in South Spain. In contrast to the scarce hydrolytic activity shown by culture collection strains, environmental isolates produce a variety of extracellular enzymes that could be of potential biotechnological interest. Identification at the genus level revealed that 25 strains and 2 out of 122 isolates belonged to *Halomonas* and *Chromohalobacter*, respectively. An  $\alpha$ -amylase produced by *Halomonas meridiana* has been studied at the biochemical and molecular level (Coronado et al., 2000a; Coronado et al., 2000b). The amylase showed optimal activity at 10% NaCl, 37°C and pH 7.0 (being relatively stable under alkaline conditions). The enzyme showed activity at high salt concentrations (up to 30%). The main products resulting from the hydrolysis of starch were maltose and maltotriose. The gene encoding this  $\alpha$ -amylase (*amyH*) has been cloned and expressed in the heterologous hosts *H. elongata* and the non-halophilic bacterium *E. coli* (Coronado et al., 2000b). It encodes a 457-residue protein with a molecular mass of 50 kDa. Besides, an extracellular  $\alpha$ -amylase gene from the hyperthermophilic archaeon *Pyrococcus woesei* has been cloned and expressed in *H. elongata*, under the control of a native *H. elongata* promoter (Frillingos et al., 2000).

Halomonads and other moderately halophilic bacteria can be also used for the degradation of toxic compounds both at high and intermediate salt concentrations, permitting the restoration of saline industrial residues and contaminated

saline environments (Mellado and Ventosa, 2003). However, few studies have been carried out. Some examples of halomonads with a potential for biodegradation and biotransformation of contaminants (such as aminomethane sulfonate, organophosphonates, benzoate, formaldehyde, phenol, selenate and uranium compounds) are shown in Table 7. Maltseva et al. (1996) isolated halomonads able to use chloroaromatic compounds as the sole source of carbon and energy and studied in detail the degradation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Kleinstaubler et al. (2001) reported the use of the alkaliphilic, moderately halophilic *Halomonas* sp. strain EF43 for the expression of the 2,4-D-degradative pathway by conjugation of the broad host range plasmid pJP4. This strain was able to degrade 2,4-D- and 3-chlorobenzoate under alkaline conditions in the presence of an additional carbon source.

Very recently, a screening of moderately halophilic bacteria able to use aromatic compounds permitted the isolation of a large number of members of the genus *Halomonas* able to degrade different compounds. Some of them may represent new species of *Halomonas*, such as "*H. organivorans*," which is able to use benzoic acid, *p*-hydroxybenzoic acid, cinnamic acid, salicylic acid, phenylacetic acid, phenylpropionic acid, phenol, *p*-coumaric acid, ferulic acid and *p*-aminosalicylic acid (M. T. García et al., personal communication). The tolerance patterns of several *Halomonas* species and *Chromohalobacter marismortui* to ten heavy metals have been studied, as well as the influence of salinity and composition of culture media (Nieto et al., 1989). These studies may be interesting for future use of metal-tolerant halophilic strains as biological detoxicants.

Few studies have been carried out on the role of halomonads in the fermentation processes of foods and other products. *Halomonas alimentaria* was isolated from the traditional Korean fermented seafood jeotgal (Yoon et al., 2002). The hydrolytic activity of *H. elongata* in bacon curing brines has been investigated (Hinrichsen et al., 1994).

Fuel ethanol production has been proposed using the pyruvate decarboxylase (PDC) of *Z. palmae* as biocatalyst (Raj et al., 2002). This enzyme is thermostable and showed the highest specific activity and lowest  $K_m$  for pyruvate of all four known bacterial PDCs.

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## The Genus *Deleya*

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The genus *Deleya*, along with the genus *Halomonas*, comprises the family Halomonadaceae (Franzmann et al., 1988). This family encompasses various halotolerant and moderately halophilic rod-like Gram-negative nonfermentative, chemoorganotrophs that require 75 mM to 200 mM NaCl for growth. However, some *Deleya* strains grow optimally only in media containing at least 7.5% salts (1.3 M NaCl). “Salt-loving” is a universal feature of all strains belonging to the genera *Deleya* and *Halomonas*, and *Deleya* strains have been isolated from marine environments, solar salterns, saline soils, and salted food.

Within the Proteobacteria, the family Halomonadaceae belongs to the rRNA superfamily II sensu De Ley (1978) (see The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition), i.e., part of the gamma subclass sensu Stackebrandt et al. (1988). The genus *Deleya* consists of six validly published species: *D. aesta*, *D. cupida*, *D. halophila*, *D. marina*, *D. pacifica*, and *D. venusta* (Baumann et al., 1983; Quesada et al., 1984). The genus is both genotypically and phenotypically heterogeneous; the GC content of the DNA ranges from 52 to 68 mol%. Good evidence exists (see below) that these six *Deleya* species, together with the *Halomonas* species, are part of a single evolutionary lineage. DNA-rRNA hybridizations (De Vos et al., 1989, and this chapter) and chemotaxonomic analyses (Akagawa and Yamasato, 1989; Franzmann and Tindall, 1990) revealed that various organisms misnamed as members of the genera *Pseudomonas*, *Alcaligenes*, and *Achromobacter* are authentic members of the genus *Deleya*. In the text below, misnamed taxa are enclosed by brackets, [ ], and invalid taxon names are in quotation marks, “ ”.

### **Intra- and Suprageneric Relationships of *Deleya* Species and Allied Bacteria**

In a taxonomic study of more than 200 Gram-negative, nonfermentative, marine eubacterial

strains, Baumann et al. (1972) assigned peritrichously flagellated strains of four different phenotypes to four new species of the genus *Alcaligenes* (*[A.] aestus*, *[A.] cupidus*, *[A.] pacificus*, and *[A.] venustus*, respectively). Hendrie et al. (1974) considered *[Achromobacter] aquamarinus* (ZoBell and Upham, 1944) as an acceptable species within the genus *Alcaligenes*. However, it is now well established that all these marine *[Alcaligenes]* strains are genotypically and phenotypically very different from the type species *Alcaligenes faecalis*. The latter species belongs, together with some other *Alcaligenes* taxa and all *Bordetella* species, in the family Alcaligenaceae (De Ley et al., 1986) within the rRNA superfamily III (beta subclass) of the Proteobacteria (see also The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition). On the other hand, all marine *[Alcaligenes]* species are members of the rRNA superfamily II (part of the gamma subclass) (De Ley, 1978; Kersters and De Ley, 1980) and are phylogenetically more related to the authentic *Pseudomonas* species (i.e., *Pseudomonas* section I of Palleroni, 1984). In *Bergey's Manual of Systematic Bacteriology*, the marine *[Alcaligenes]* species were therefore considered as species incertae sedis (Kersters and De Ley, 1984). Evolutionary studies on pathways of carbohydrate metabolism (Sawyer et al., 1977) and on patterns of regulation of aspartokinase activities (Baumann and Baumann, 1974), immunological studies on superoxide dismutases and glutamine synthetases (Baumann and Baumann, 1978; DeLong et al., 1984) as well as chemotaxonomic data (Akagawa and Yamasato, 1989; Franzmann and Tindall, 1990), also yielded strong evidence that the peritrichously flagellated marine *[Alcaligenes]* species constitute a single evolutionary lineage, together with the polarly flagellated species *[Pseudomonas] marina*. This group of bacteria is unrelated to the authentic *Alcaligenes* species and is also distinct from the fluorescent pseudomonads, which are their close relatives. On the basis of all these arguments Baumann et al. (1983) proposed a new genus called *Deleya* to accommodate the marine *[Alcaligenes]* species together with *[P.] marina*. *D. halophila* was described by Quesada et al. (1984) for strains isolated from hypersaline soils in Spain. On the

basis of cellular fatty acid profiles, biochemical and physiological features and DNA-DNA hybridizations, Akagawa and Yamasato (1989) proposed that [*Alcaligenes*] *aquamarinus* was synonymous with *Deleya aesta* and *Deleya aquamarina* be recognized as the type species of the genus *Deleya*. DNA-rRNA hybridizations were performed in order to unravel the inter- and intrageneric relationships of the species of the genus *Deleya*. Fig. 1 summarizes our present knowledge of the position of various taxa and strains on the *Deleya* rRNA branch. All hybridizations were carried out versus labeled rRNA of *Deleya aquamarina* NCMB 557<sup>T</sup> (the former [*Alcaligenes*] *aquamarinus*). The branching point of the genus *Halomonas* at a Tm(e) value of 76°C agrees very well with data from 16S rRNA cataloging (Franzmann et al., 1988). With the latter method, four halophilic species, *Halomonas elongata*, *H. subglaciescola*, *H. halmophila* (ex [*Flavobacterium*] *halmophilum*; Franzmann et al., 1988) and *D. aesta*, form a fairly coherent cluster phylogenetically, with a lowest branching at S<sub>AB</sub> 0.60 (Franzmann et al., 1988). In the Tm(e) dendrograms, the family Halomonadaceae at 70°C is closest to the authentic *Pseudomonas* group and to *Marinomonas* (Fig. 1).

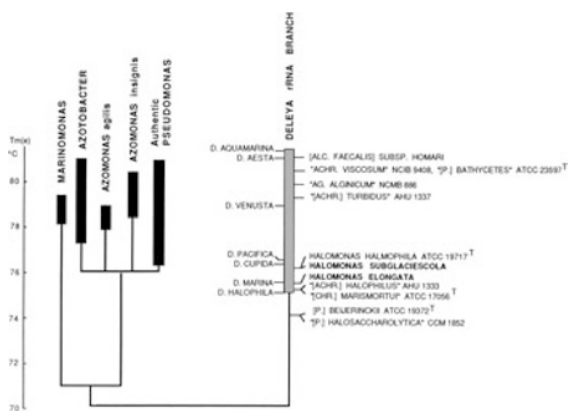


Fig. 1. Simplified rRNA cistron-similarity dendrogram of *Deleya* and related taxa. Tm(e) is the temperature at which 50% of the DNA-rRNA hybrid is eluted. The black bars at the top of the branches represent the ranges of the Tm(e) values of the reference taxa. The position of all validly published *Deleya* species is indicated at the left side of the *Deleya* rRNA branch, whereas three species of *Halomonas* and various generically misnamed taxa and strains are on the right side of this rRNA branch. Abbreviations: ACHR., *Achromobacter*; AG., “*Agarbacterium*”; ALC., *Alcaligenes*; CHR., *Chromobacterium*; D., *Deleya*; F., *Flavobacterium*; P., *Pseudomonas*. The position of this group of bacteria within rRNA superfamily II (part of the gamma subclass) of the Proteobacteria can be found in Fig. 5 of The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition. Tm(e) values were taken from De Vos and De Ley (1983), De Vos et al. (1989), Van Landschoot and De Ley (1983), and P. Segers (unpublished observations).

On the basis of Tm(e) values of DNA-rRNA hybrids versus *D. aquamarina* NCMB 557<sup>T</sup>, two groups of strains can be recognized on the *Deleya* rRNA branch (Fig. 1):

1. Strains located in the top 3°C ΔTm(e), including *D. aquamarina* (the type species), *D. aesta* (synonym of *D. aquamarina*), *D. venusta*, [*A. faecalis*] subsp. *homari* isolated from lobsters, “[*P.*] *bathycetes*” “*Agarbacterium alginicum*,” and some misnamed *Achromobacter* strains; all these strains possess DNA with a GC content of 52–61 mol%. Akagawa and Yamasato (1989) found that [*A. faecalis*] subsp. *homari* is highly similar to *D. aquamarina*. When more data on their phenotype and genotype will be available, the other misnamed strains will very likely be allocated to the genus *Deleya* either as synonyms of existing species or as separate species.

2. Strains located at the bottom 5–7°C ΔTm(e), including *D. cupida*, *D. halmophila*, *D. marina*, *D. pacifica*, *H. elongata* (type species of the genus *Halomonas*), *H. subglaciescola*, and various misnamed strains (see Fig. 1); the GC content of their DNA ranges from 59 to 68 mol%. More detailed rRNA comparisons are required to elucidate the exact relationships between the different members of this group. In particular, the relationships among this group of strains and all members of the genus *Halomonas* need to be investigated.

### Generically Misnamed Taxa and Strains of the *Deleya* rRNA Branch

Genomic and phenotypic studies have indicated that a great number of generically misnamed taxa and strains are authentic members of the *Deleya* rRNA branch (P. Segers and K. Kersters, unpublished observations). The position of these taxa and individual strains is indicated in Fig. 1; additional information on the habitats of these organisms can be found in Table 1.

“[*Pseudomonas*] *bathycetes*” originates from deep-sea sediments (Quigley and Colwell, 1968); “*Agarbacterium alginicum*” was isolated from the surface of brown algae and [*Alcaligenes faecalis*] subsp. *homari* is pathogenic for lobsters (Austin et al., 1981). The immunological data of DeLong et al. (1984) on glutamine synthetases indicate that [*Alcaligenes faecalis*] subsp. *homari* is not related to *Alcaligenes*, but it is highly similar to *Deleya*. DNA-rRNA hybridizations (Fig. 1) show that these taxa, together with strains “[*Achr.*] *viscosum*” NCIB 9408 and “[*Achr.*] *turbidus*” AHU 1337, belong to the genus *Deleya*. Some misnamed [*Pseudomonas*] and [*Achromobacter*] strains were isolated from salted food, whereas “[*Chromobacterium*] *marismortui*” originates from water of the Dead Sea.

Table 1. Sources and geographical distribution of taxa and strains belonging to the *Deleya* rRNA branch.

Species or strain <sup>a</sup>	Source of isolation	Geographical origin	Reference
<i>Deleya aesta</i>	Seawater (at 100–600 m deep)	Hawaii, USA	Baumann et al. (1972)
<i>Deleya aquamarina</i> (ex [ <i>Alcaligenes</i> ] <i>aquamarinus</i> )	Seawater	Hawaii, USA	ZoBell and Upham (1944)
<i>Deleya cupida</i>	Seawater (surface)	Hawaii, USA	Baumann et al. (1972)
<i>Deleya halophila</i>	Hypersaline soil	Spain	Quesada et al. (1983, 1984)
<i>Deleya marina</i>	Seawater (surface)	Hawaii, USA	Baumann et al. (1972)
<i>Deleya pacifica</i>	Seawater (surface)	Hawaii, USA	Baumann et al. (1972)
<i>Deleya venusta</i>	Seawater (surface)	Hawaii, USA	Baumann et al. (1972)
<i>Halomonas elongata</i>	Solar saltern	Netherlands Antilles	Vreeland et al. (1980)
<i>Halomonas subglactescola</i>	Hypersaline lake	Organic Lake, Antarctica	Franzmann et al. (1987)
<i>Halomonas halmophila</i> ATCC 19717 <sup>T</sup>	Hypersaline lake	Dead Sea, Israel	Elazari-Volcani (1940)
(ex “[ <i>Flavobacterium</i> ] <i>halmophilum</i> ”)			
“[ <i>Achromobacter</i> ] <i>halophilus</i> ” AHU 1333	Salted salmon	Japan	Bergey et al. (1930)
“[ <i>Achromobacter</i> ] <i>turbidus</i> ” AHU 1337	Salted eggplant	Japan	
“[ <i>Achromobacter</i> ] <i>viscosum</i> ” NCIB 9408			
“ <i>Agarobacterium alginicum</i> ” NCMB 886			
[ <i>Alcaligenes faecalis</i> ] subsp. <i>homari</i>	Mass of brown algae	Georgia, USA	Bergey et al. (1925)
“[ <i>Chromobacterium</i> ] <i>marismortui</i> ” ATCC 17056 <sup>T</sup>	Hemolymph of moribund lobsters	Mass., USA	Adams et al. (1961), Eller and Payne (1960)
“[ <i>Pseudomonas</i> ] <i>bathycetes</i> ” ATCC 23597 <sup>T</sup>	Hypersaline lake	Dead Sea, Israel	Austin et al. (1981)
[ <i>Pseudomonas</i> ] <i>bejerinckii</i> ATCC 19372 <sup>T</sup>	Deep-sea sediments (at > 9,000 m)	Pacific Ocean	Elazari-Volcani (1940)
“[ <i>Pseudomonas</i> ] <i>halosaccharolytica</i> ” CCM 2851	Salted French beans		Quigley and Colwell (1968)
	Rock salt	Japan?	Hof (1935), Breed et al. (1957)
			Ohno et al. 1979, Yamada and Shiio (1953)

<sup>a</sup>Abbreviations: AHU, Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo, Japan; ATCC, American Type Culture Collection, Rockville, Md., USA; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland; NCMB, National Collection of Marine Bacteria, Torry Research Station, Aberdeen, Scotland.



## Intragenetic Relationships of Species of *Deleya*

Data on the finer internal taxonomic relationships of the genus *Deleya* are scarce. Most of the well-established *Deleya* species have been delineated on the basis of numerical taxonomic studies of phenotypic features (Baumann et al., 1972; Marquez et al., 1987; Quesada et al., 1983). DNA-DNA hybridizations revealed that the following taxa are highly related (more than 75% homology): *D. aesta*, *D. aquamarina*, the lobster isolates (*[A. faecalis]* subsp. *homari*), and “[*Achromobacter*] *viscosum*” strain NCIB 9408 (P. Segers and B. Hoste, unpublished observations). They are all located in the top 1°C ΔT<sub>m</sub>(e) of the *Deleya* rRNA branch (Fig. 1), and the GC content of their DNA ranges from 57 to 61 mol%. No significant DNA homologies were detected between *D. aquamarina* and the type strains of the other *Deleya* species (Akagawa and Yamasato, 1989).

## Habitats

One common characteristic of all the strains belonging to the *Deleya* rRNA branch is their isolation from salt-containing materials. Table 1 gives an overview of the various habitats where strains of the *Deleya* rRNA branch have been found. Many *Deleya* strains are from marine origin and grow optimally at salt concentrations of 0.4% to 1.2% NaCl (Baumann et al., 1983), but others such as *D. halophila* are moderate halophiles because they display optimal growth at 7.5% NaCl (Ferrer et al., 1987). *D. halophila* strains were usually isolated from hypersaline soils and solar salterns (Quesada et al., 1982; 1983). *Deleya* strains have also been isolated from ponds of an inland Spanish saltern which was supplied with water from a subterranean inland saline well (del Moral et al., 1987, 1988).

Some strains—previously assigned to the ill-defined genus *Achromobacter*—were isolated from various salt-containing foods (Table 1). Austin et al. 1981 isolated [*Alcaligenes faecalis*] subsp. *homari* strains from the hemolymph of moribund and dead lobsters. These lobsters were reared in tanks containing seawater; their death occurred after the water in the tanks accidentally reached a temperature of 26°C for three days. The isolated bacteria were found to be pathogenic for lobsters. It has been proven (see above) that three of these isolates (including the type strain) were phenotypically and genomically highly related to *D. aesta* (type species) and *D. aquamarina* (Fig. 1).

## Isolation

The following media and isolation procedures were used by several authors for the isolation of

strains belonging to the *Deleya* rRNA branch. More details can be found in Baumann and Baumann (1981).

## Media For the Isolation of *Deleya* Species

### Artificial Seawater (ASW) (MacLeod, 1968)

NaCl	400 mM
MgSO <sub>4</sub> · 7H <sub>2</sub> O	100 mM
KCl	20 mM
CaCl <sub>2</sub> · 2H <sub>2</sub> O	20 mM

Dissolve the salts separately in distilled water and combine.

### Basal Medium (BM)

TrisHCl (pH 7.5)	50 or 100 mM
NH <sub>4</sub> Cl	19 mM
K <sub>2</sub> HPO <sub>4</sub> · 3H <sub>2</sub> O	0.33 mM
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.1 mM
Half-strength ASW	

The carbon source is usually supplied at a concentration of 0.1–0.2% (w/v for solids and v/v for liquids). Labile compounds should be filter-sterilized.

### Basal Medium Agar (BMA)

Prepare equal volumes of double-strength BM and double-strength agar in distilled water (40 g agar per liter), sterilize by autoclaving, and combine before pouring on plates. Carbon and energy sources can be added to double-strength BM prior to autoclaving. Labile compounds should be filter-sterilized and added to BMA, which has been cooled to about 42°C prior to pouring on plates.

### Marine Agar (MA)

Marine agar 2216E is available from Difco Laboratories, Detroit, Mich., USA.

### Medium for Moderately Halophilic Bacteria (MH Medium) (Quesada et al., 1983)

5 g proteose peptone, 10 g yeast extract, and 1 g glucose are dissolved in 10% (w/v) marine salts solution. Marine salts solution contains, in one liter of distilled water:

NaCl	81 g
MgCl <sub>2</sub> · 6H <sub>2</sub> O	7 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	9.6 g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.36 g
KCl	2 g
NaHCO <sub>3</sub>	0.06 g
NaBr	0.026 g

The pH is adjusted to 7.2 with 1 M KOH, and agar (20 g/liter) is added.

MH medium with e.g., 20% marine salts, is prepared by doubling the amount of inorganic salts in the above medium. Lowering the concentration of Mg<sup>2+</sup> (to 1 g MgSO<sub>4</sub> · 7H<sub>2</sub>O/liter) increases the selectivity of the MH medium for moderate halophiles, because growth of extreme halophiles is suppressed (Marquez et al., 1987).

## Isolation Procedures

ISOLATION FROM SEAWATER (BAUMANN ET AL., 1972) The enrichment culture methods have been described by Baumann et al. (1971) and Baumann and Baumann (1981). Various amino

acids (L-lysine, L-glutamate, L-histidine), amines (e.g., histamine) and organic acids (glycolate, *o*-hydroxybenzoate) have been used as sole carbon source for enrichment of *Deleya* strains (Baumann et al., 1972). The strains are purified on BMA containing 0.1% of the same carbon compound as used in the enrichment, or on BMA containing 0.1% Na succinate, 0.1% Na lactate and 0.1% Na acetate.

For direct isolation, samples of sea water are filtered through 0.22- or 0.45- $\mu\text{m}$  pore size nitrocellulose filters, which are placed in petri dishes containing Marine Agar or Basal Medium Agar with 0.1% organic substrates (e.g., *meso*-inositol, D-galactose, adipate, L-valine, etc.) (Baumann et al., 1972).

**ISOLATION FROM HYPERSALINE SOILS** (QUESADA ET AL., 1983, 1984) *D. halophila* was isolated from hypersaline soils in Spain by suspending one soil sample in 10 ml of 10% marine salts solution (see above); 10-fold dilutions were made (always keeping the same balanced salt concentrations), and 0.1 ml of each dilution was plated on MH medium.

**ISOLATION FROM THE HEMOLYMPH OF LOBSTERS** (AUSTIN ET AL., 1981) Drops of aseptically collected hemolymph from moribund lobsters (*Homarus americanus*) were inoculated on Marine Agar medium for the isolation of [*Alcaligenes faecalis*] subsp. *homari*. Plates were incubated at 19°C for 7 days.

**PRESERVATION OF CULTURES** Baumann and Baumann (1981) recommended maintenance of *Deleya* strains on MA slants. After each monthly transfer, cultures were allowed to grow at 25°C for 1–2 days and stored at 18°C. Quesada et al. (1983) maintained their strains on slants of MH medium. We maintained *Deleya* strains at 4°C on slants of the following medium: 1% peptone, 0.8% beef extract powder, 3% NaCl, and agar in tap water. Lyophilization is recommended for long-term preservation.

## Identification

The range of GC content of the DNAs of members of the genus *Deleya* is very broad (52–68 mol%) and is reflected in 1) the fairly great span of  $\Delta T_m(e)$  values of their DNA-rRNA hybrids versus labeled rRNA of *D. aquamarina* (see Fig. 1); and 2) considerable phenotypic and genotypic heterogeneity within the genus. An important common feature for all *Deleya* species is their absolute requirement for at least 75–200 mM NaCl for optimal growth. This feature differentiates *Deleya* from terrestrial *Pseudo-*

*monas* and *Alcaligenes* species (Baumann and Baumann, 1981).

*Deleya* species can be differentiated from other marine eubacteria by four important features: absence of fermentation of D-glucose, accumulation of poly- $\beta$ -hydroxybutyrate, peritrichously flagellated cells (except *D. marina*), and by the parameters of their DNA-rRNA hybrids. Differentiation of *Deleya* from *Halomonas* species is difficult because no comprehensive phenotypic or genomic studies have been performed using standardized procedures. Species of *Deleya* can best be differentiated from each other by carbon assimilation tests (Table 2).

*Deleya* strains are Gram-negative straight rods, 0.8 to 1.1  $\mu\text{m}$  wide and 1.5 to 3.0  $\mu\text{m}$  long. They accumulate poly- $\beta$ -hydroxybutyrate as an intracellular reserve product. Involution forms may occur in old cultures or under adverse conditions of cultivation. Endospores are not formed. Motile by four to twelve peritrichous flagella, except *D. marina* which is motile by two to five polar flagella. They are obligately aerobic, possessing a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Colonies are nonpigmented. They do not denitrify or fix molecular nitrogen and do not grow chemolithotrophically with hydrogen gas and CO<sub>2</sub>. All species are chemoorganotrophs able to grow on a mineral medium containing sea water base, D-glucose and NH<sub>4</sub>Cl. Na<sup>+</sup> is an absolute requirement for all species; the minimal concentration for optimal growth ranges from 75 to 200 mM (or even 1.3 M for *D. halophila*). All species utilize acetate, succinate, DL- $\beta$ -hydroxybutyrate, DL-lactate, glycerol, and L- $\alpha$ -alanine. All *Deleya* (and *Halomonas*) strains contain ubiquinone 9 as the major respiratory quinone with traces of Q8, and 16:1 *cis* 9, 16:0, 17:0 cyclo, 18:1 and 19:1 cyclo 11–12 as major fatty acids (Franzmann and Tindall, 1990). The GC content of the DNA ranges from 52 to 68 mol%. The type species is *D. aquamarina* (type strain ATCC 14400, NCMB 557, DSM 30161, LMG 2853, IAM 12550).

Table 2 lists the diagnostic features for the differentiation of species of the genus *Deleya*. The original publications should be consulted for full descriptions (see references in footnotes of Table 2). The type strains of the species are also listed at the bottom of Table 2. Akagawa and Yamasato (1989) give a few other biochemical and physiological features to differentiate the type strains of the *Deleya* species.

## Physiological Properties

Species of *Deleya* have no organic growth factor requirements and utilize a great variety of organic compounds as sole sources of carbon and

Table 2. Features differentiating the species of *Deleya*.<sup>a</sup>

Feature	<i>D. aestiv</i> <sup>b</sup> (n = 6)	<i>D. aquamarina</i> <sup>c</sup> (n = 1)	<i>D. cupida</i> <sup>d</sup> (n = 5)	<i>D. halophila</i> <sup>d</sup> (n = 38)	<i>D. marina</i> <sup>b</sup> (n = 7)	<i>D. pacifica</i> <sup>b</sup> (n = 6)	<i>D. venusta</i> <sup>b</sup> (n = 14)
Flagellation	pr	pr	pr	pr	p	pr	pr
Oxidase	+	+	-	+	-	+	+
Growth at 4°C	-	-	-	+	[+]	-	+
Growth on:							
L-Arabinose	-	-	+	+	-	-	-
D-Ribose	-	+	[+]	+ <sup>e</sup>	+	-	-
D-Mannose	-	-	+	+	-	-	-
Saccharate	-	+	+	- <sup>e</sup>	-	-	-
Suberate	+	+	-	+ <sup>e</sup>	-	-	-
Glycolate	-	-	+	- <sup>e</sup>	-	-	-
Aconitate	-	+	+	[+]	+	[+]	[+]
Mannitol	+	+	+	[+]	+	-	[+]
δ-Aminovalerate	-	-	[+]	-	-	+	+
L-Histidine	-	-	[+]	-	-	+	+
L-Tyrosine	-	-	+	+ <sup>e</sup>	+	+	+
DL-Kynurenine	-	-	-	- <sup>e</sup>	-	+	-
Ethanolamine	-	-	-	- <sup>e</sup>	-	-	+
Putrescine	-	-	+	- <sup>e</sup>	-	+	+
Sarcosine	-	-	+	- <sup>e</sup>	[+]	+	+
Ring cleavage	-	-	o	-	-	o	o
GC content (mol%)	57-58	58	60-63	67	62-64	67-68	52-55
Type strain no.	ATCC 27128	ATCC 14400	ATCC 27124	CCM 3662	ATCC 25374	ATCC 27122	ATCC 27125

<sup>a</sup>Symbols: n, number of strains used; pr, peritrichous; p, polar; +, all strains positive; [-], more than 80% of strains positive; -, all strains negative; o, ortho-cleavage of aromatic ring for strains capable of growth on aromatic compounds.

<sup>b</sup>Data from Baumann et al. (1972, 1983).

<sup>c</sup>Data from Kersters and De Ley (1984).

<sup>d</sup>Data from Quesada et al. (1984).

<sup>e</sup>Determined with auxanographic API galleries (API System, Montalieu Vercieu, France) for the type strain only.

energy. They can assimilate pentoses, hexoses, disaccharides, sugar alcohols, sugar acids, fatty acids, dicarboxylic acids, tricarboxylic acid cycle intermediates, amino acids, amines, and some aromatic substances. The latter compounds are metabolized via the  $\beta$ -keto adipate pathway involving the *ortho*-cleavage of diphenolic compounds (Baumann et al., 1972). D-Glucose and D-fructose are metabolized via the Entner-Doudoroff pathway (Baumann and Baumann, 1973; De Ley et al., 1970; Sawyer et al., 1977).

Growth characteristics of the type strain of *Deleya halophila* have been studied in more detail by the research group of A. Ramos-Cormanzana. *D. halophila* displays a specific requirement for  $\text{Na}^+$ , which cannot be replaced by other cations (Quesada et al., 1987). Optimal growth of *D. halophila* occurs at 7.5% total salts, when incubated at 32 or 42°C. However, when the incubation temperature is lowered to 22°C, its optimal growth occurs at 5% total salts. *D. halophila* is able to grow at salt concentrations varying from 2.5% to 25% (Ferrer et al., 1987). Such characteristics are typical of moderately halophilic bacteria isolated from hypersaline soils, where salt content can vary considerably in space and time.

Investigations have been performed on the effects of growth temperature and salt concentration on the cellular fatty acid composition of *D. halophila*, "[*Pseudomonas*] *halosaccharolytica*," and *Halomonas halmophila* (ex [*Flavobacterium*] *halmophilum*; Franzmann et al., 1988) (Monteoliva-Sanchez and Ramos-Cormenzana, 1986, 1987; Monteoliva-Sanchez et al., 1988; Ohno et al., 1979). Increasing the salt concentration in the medium resulted in an increase of cyclopropanoic acids with a concomitant decrease in the monounsaturated fatty acids. [*Pseudomonas*] *beijeinckii* and *Halomonas halmophila* mainly contained ubiquinone Q-9 (Collins et al., 1981).

"*Agarbacterium alginicum*" hydrolyzes agar, a property which can be lost upon continued laboratory culture. The alginase of this bacterium has been studied in some detail (Williams and Eagon, 1962).

Calvo et al. 1988 were the first authors to report the existence of bacteriophages active against moderately halophilic bacteria. They demonstrated the existence of temperate phages in 52% of their *D. halophila* strains. Phage F9-11 (isolated from *D. halophila* strain F9-11) was studied in more detail and possessed an isometric head and a noncontractile tail. The phage could replicate at a wide range of marine salt concentrations, from 2.5% to 15%. Its stability seemed to be influenced by the osmolarity of the medium rather than by the NaCl concentration (Calvo et al., 1988).

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## The Genus *Frateuria*

JEAN SWINGS

The genus *Frateuria* comprises bacteria formerly classified as *Acetobacter aurantius* (Kondo and Ameyama, 1958). These are strains isolated from the plant *Lilium auratum*, which are brown, pigment-producing, polarly-flagellated, acetic acid-like bacteria. They were thought by Asai (1968) to be an evolutionary “intermediate” between *Pseudomonas* and *Gluconobacter*, and the position of these “intermediate” strains was discussed in a separate chapter. Within the acetic acid bacteria, their assignment either to *Gluconobacter* or *Acetobacter* has been difficult (Swings et al., 1980; Yamada et al., 1976). Since DNA-rRNA hybridization studies showed that these isolates are quite removed from the family *Acetobacteraceae*, the new genus *Frateuria* was created for them (Swings et al., 1980). *Frateuria* belongs in the Proteobacteria in subclass gamma sensu Stackebrandt et al. (1988) (see The proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition). In *Bergey’s Manual of Systematic Bacteriology*, *Frateuria* was treated as a separate genus (Swings et al., 1984).

### Habitats

Only two habitats of *Frateuria* are known, *Lilium auratum* (Kondo and Ameyama, 1958) and the fruit of the raspberry *Rubus parvifolius* (Yamada et al., 1976). Only 14 strains are known and described in the literature.

### Isolation

Isolation from Raspberries (*Rubus parvifolius*) (Yamada et al., 1976)

The picked raspberries were incubated at 30°C in a medium containing glucose, 10 g; ethanol, 5 ml; yeast extract, 5 g; peptone, 3 g; and acetic acid 0.3 ml, per liter of 10% potato extract adjusted to pH 4.5. After the enrichment culture, typical acetic acid-like bacteria were selected on CaCO<sub>3</sub> plates, (see The Genera *Acetobacter* and *Gluconobacter* in the second edition), where they dissolved the calcium carbonate.

### Identification

As *Frateuria* possesses the phenotypical features of the family Acetobacteraceae (see The Genera *Acetobacter* and *Gluconobacter* in the second edition), it was initially classified as an acetic acid bacterium. The minimal phenotypic description is as follows: Gram-negative, strictly aerobic, polarly flagellated rods when motile, requiring no growth factors, producing a water-soluble brown pigment on glucose-yeast extract-CaCO<sub>3</sub> agar, producing ubiquinone (menaquinone) with eight isoprenoid units (MK-8) and H<sub>2</sub>S, able to grow at pH 3.6 and on Frateur’s Hoyer mannitol medium; producing acid from ethanol, glucose, and xylose; lacking oxidase and gelatinase; not reducing nitrates; not hydrolyzing starch.

*Frateuria* presents another example of the unreliability of using solely phenotypic characterizations at the inter- and suprageneric levels in bacteriology. The production of acetic acid from ethanol and the growth at pH 3.6 suggest a relationship with the acetic acid bacteria which is not justified by more detailed phenotypic analysis. Additional methods are necessary in order to identify the genus *Frateuria* unambiguously, e.g., the types of ubiquinones formed, cellular fatty acid composition and DNA-rRNA hybridization analysis. *Frateuria* typically produces the ubiquinone MK-8, whereas *Gluconobacter* produces MK-10 and *Acetobacter* MK-9 or MK-10 (Yamada et al., 1976). *Frateuria* has an unusual fatty acid profile, consisting of the iso-branched-chain acid of C<sub>15:0</sub>, which is different from those of *Acetobacter* or *Gluconobacter* (Yamada et al., 1981) and which is more closely related to *Xanthomonas maltophilia*. The application of DNA-rRNA hybridizations constituted the ultimate proof that *Frateuria* is not related to the acetic acid bacteria (subclass alpha of the Proteobacteria), but constituted a separate genus within subclass gamma. The acid-tolerant, nitrogen-fixing bacterium isolates from sugarcane resembled *Frateuria* by its production of a brown diffusible pigment and of H<sub>2</sub>S, its lack of growth factor requirements, and its growth on 30% glucose (Cavalcante and Döbereiner, 1988), but it could be assigned unambiguously to the

genus *Acetobacter* by DNA-rRNA hybridizations (Gillis et al., 1989) (see The Genera *Acetobacter* and *Glucenobacter* in the second edition). The genus *Frateuria* currently comprises only one species, *Frateuria aurantia* (Swings et al., 1980, 1984).

## Physiological Properties

Good growth occurs on glucose-yeast extract-CaCO<sub>3</sub> agar and in beer wort. In peptone broth, yeast extract broth, and Hoyer's mannitol medium, growth is faint. In Hoyer's medium, NH<sub>4</sub><sup>+</sup> is used as a nitrogen source in the presence of mannitol as a carbon source. All *Frateuria* strains grow well on casamino acids, in contrast with the acetic acid bacteria. Growth factors are not required.

The oxidation of lactate was demonstrated on oxydograms. In an oxydogram (Frateur, 1950), several strains are streaked on 2% agar plus 2% Ca lactate. Lactate oxidation is shown by the precipitation of CaCO<sub>3</sub>. The ketogenic activity on polyalcohols was weak or doubtful. From glucose, 2-keto-, and 2,5-diketogluconic acids were formed but not 5-ketogluconic acid. The production of acid from D-glucose and D-xylose was marked, and the pH always dropped below 4. Ethanol, glycerol, D-arabinose, D-ribose, D-fructose, D-galactose, and D-mannose were also acidified (Swings et al., 1980). Ethanol concentrations above 5% were not tolerated. The majority of the strains still grew in 25 to 30% glucose concentrations, and, in this respect, they also resemble the acetic acid bacteria.

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# The Chromatiaceae

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## Introduction

The Chromatiaceae are  $\gamma$ -Proteobacteria (Woese et al., 1985) and representatives of the phototrophic purple bacteria. They are also referred to as “purple sulfur bacteria” (together with the Ectothiorhodospiraceae) and typically grow under anoxic conditions in the light using as photosynthetic electron donor, sulfide, which is oxidized to sulfate via intermediate accumulation of elemental sulfur inside the cells. A number of species also can grow under chemotrophic conditions in the dark, either autotrophically or heterotrophically using oxygen as terminal electron acceptor in respiratory processes. Phototrophic growth, photosynthetic pigment synthesis, and internal membrane formation are strictly regulated by oxygen and become derepressed at low oxygen tension.

## Phylogeny

The family Chromatiaceae comprises physiologically similar species and genera of the  $\gamma$ -Proteobacteria that carry out anoxygenic photosynthesis (Fowler et al., 1984; Guyoneaud et al., 1998; Imhoff et al., 1998). The Chromatiaceae form a well-defined phylogenetic group of the  $\gamma$ -Proteobacteria to which at present none of the purely chemotrophic bacteria is closely related. They are closely related to the Ectothiorhodospiraceae, a second family of the purple sulfur bacteria (Imhoff, 1984b). Both are phylogenetically distant from the phototrophic purple nonsulfur bacteria, which are  $\alpha$ - and  $\beta$ -Proteobacteria.

The 16S rDNA nucleotide sequences of most Chromatiaceae species are available and form the basis for a new taxonomy of this family (Cauette et al., 1997; Guyoneaud et al., 1998; Imhoff et al., 1998). Positive correlation between similarities of 16S rDNA sequences and particular phenotypic properties were taken as an indication of the importance of these properties in achieving a phylogenetically oriented taxonomy

that includes both genetic and phenotypic information for classification (Imhoff et al., 1998). It was suggested that the salt response is one important taxonomic criterion in such a taxonomic system of the Chromatiaceae, because both the genetic relationship and the salt responses distinguish major phylogenetic branches of the Chromatiaceae and single genera. Both the genetic relationship and the salt responses enable, e.g., the halophilic *Halochromatium salexigens* and *Halochromatium glycolicum*, and the marine *Marichromatium gracile* and *Marichromatium purpuratum* to be distinguished from each other and from freshwater species such as *Allochromatium vinosum* and relatives (Table 1 and Fig. 1). This implies a separate evolutionary development in the marine and in the freshwater environment and points to the general importance of salt responses and possibly other ecological parameters for species formation and evolution (Imhoff, 2001a).

The genetic relatedness determined on the basis of 16S rDNA nucleotide sequences (as shown in Fig. 1) revealed that major phylogenetic branches of the Chromatiaceae contain 1) truly marine and halophilic species, 2) species that are motile by polar flagella, do not contain gas vesicles, and are primarily freshwater species, and 3) species with ovoid to spherical cells, the majority of which are nonmotile freshwater species containing gas vesicles. A number of species have considerably deep branching points in the phylogenetic tree, which depicts their genetic difference from all other bacteria within this group. Among these is *Isochromatium buderi* belonging to the group of marine species and *Thiolamprovum pedioforme*, which is clearly but distantly associated to the group of *Thiocapsa* species. To these species also belongs *Thiorhodococcus minor*, which shows variable association even to different groups if different methods are applied for the tree calculations. In the presented distance tree, *Thiorhodococcus minor* is associated to *Thiobaca trueperi*.

The marine branch includes the genera *Marichromatium*, *Halochromatium*, *Rhabdochromatium*, *Thiococcus*, *Thioflaviccoccus*,



Table 1. Selected characteristics of genera and species of the Chromatiaceae.

Genera species	motile	gas vesicles	cell form and size [ $\mu\text{m}$ ]	optimum salinity	Topt [ $^{\circ}\text{C}$ ]	vitamins required	G+C content [mol%]	chemolithotrophy	carotenoid group
<i>Chromatium</i>	m	no	rod	none	20–35	B <sub>12</sub>	48.0–50.0	no	ok
<i>Chr. okenii</i>			4.5–6.0	none	20–35	B <sub>12</sub>	48.0–50.0	no	ok
<i>Chr. weissii</i>			3.5–4.0	none	20–35	B <sub>12</sub>	48.0–50.0	no	ok
<i>Allochromatium</i>	m	no	rod	o	30–35	none	61.3–66.3	yes	sp
<i>Alc. vinosum</i>			2.0	none	30–35	none	63.7	yes	sp
<i>Alc. minutissimum</i>			1.0–1.2	none	25–30	B <sub>12</sub>	55.1–60.2	no	ra
<i>Alc. warmingii</i>			3.5–4.0	none	48–50	none	61.5	no	sp
<i>Thermochromatium</i>	m	no	rod	none	30	none	61.3	yes	ok
<i>Tch. tepidum</i>	m	no	1–2	none	30	none	62.2	yes	ok
<i>Thiocystis</i>	m	no	sphere/rod	none	25–35	none	62.8–67.9	yes	ra
<i>Tes. gelatinosa</i>			3.0	none	30–35	none	61.8–64.3	yes	ra
<i>Tes. minor</i>			2.0	o	20–35	none	63.3–66.3	yes	sp
<i>Tes. violacea</i>			2.5–3.5	o	20–35	B <sub>12</sub>	64.3	yes	sp
<i>Tes. violascens</i>			2.0	o	30	B <sub>12</sub>	65.3	no	sp
<i>Thiocapsa</i>	no		sphere	1%	30	B <sub>12</sub>	64	yes	sp
<i>Tca. roseopersicina</i>		no	1.2–3.0	o	37	none	65.5	yes	sp
<i>Tca. rosea</i>		GV	2.0–3.0	none	20–30	none	63.8	no	ra (la, lo)
<i>Tca. pendens</i>		GV	1.5–2.0	none	23–25	o	63.5	yes	ok
<i>Tca. litoralis</i>		no	1.5–2.5	none	23	none	62.5–63.5	no	ok
<i>Thiolamprovirus</i>	no	GV	sphere	none	20–25	none	65.3	no	ra
<i>Tlp. pedioforme</i>			2.0	none	20–30	none	66.3	no	ra
<i>Lamprocystis</i>			sphere	none	20–30	none			
<i>Lpc. roseopersicina</i>	m	GV	3.0–3.5	none	23–25	o			
<i>Lpc. purpurea</i>	no	GV	1.9–2.3	none	23	none			
<i>Thiopedia</i>	no	GV	sphere	none	20–25	none			
<i>Tpd. rosea</i>		GV	2.0–2.5	none	20–30	none			
<i>Thiodictyon</i>	no	GV	rod	none	20–30	none			
<i>Tdc. elegans</i>			1.5–2.0	none	20–30	none			
<i>Tdc. bacillosum</i>			1.5–2.0	none	20–30	none			

(Continued)

Table 1. Continued

Genera species	motile	gas vesicles	cell form and size [ $\mu\text{m}$ ]	optimum salinity	Topt [ $^{\circ}\text{C}$ ]	vitamins required	G+C content [mol%]	chemolithotrophy	carotenoid group
<i>Thiobaca</i>	m	no	rod	none	25–30	o	62.9–63.9	o	ly
<i>Thb. trueperi</i>	m	no	1.6	none	25–30	o	62.9–63.9	o	ly
<i>Thiorhodococcus</i>	m	no	sphere	none	25–30	o	62.9–63.9	o	ly
<i>Trc. minor</i>	m	no	1.0–2.0	2%	30–35	none	66.9	yes	sp
<i>Marichromatium</i>	m	no	rod	2–3%	30–35	none	68.9–70.4	yes	sp
<i>Mch. gracile</i>	m	no	1.0–1.3	5%	25–30	o	68.4–68.9	no	ok
<i>Mch. purpuratum</i>	m	no	1.2–1.7	5%	25–30	o	68.4–68.9	no	ok
<i>Thiococcus</i>	no	no	sphere	bchl, ICM as tubules	25	none	69.4–69.9	no	ts
<i>Tco. pfennigii</i>	m	no	1.2–1.5	1–2%	25	none	69.4–69.9	no	ts
<i>Thioflavococcus</i>	m	no	sphere	bchl, ICM as tubules	20–30	none	66.5	no	ts
<i>Tfl. mobilis</i>	(m)	no	0.8–1.0	2%	20–30	none	66.5	no	ts
<i>Thioalkalicoccus</i>	(m)	no	sphere	bchl, ICM as tubules	20–25	none	63.6–64.8	no	ts
<i>Tal. limnaeus</i>	m	no	1.3–1.8	5%	20–25	none	63.6–64.8	no	ts
<i>Rhabdochromatium</i>	m	no	rod	1.5–5%	30	none	60.4	no	ly
<i>Rbc. marinum</i>	m	no	1.5–1.7	1.5–5%	30	none	60.4	no	ly
<i>Thiorhodovibrio</i>	m	no	Spiral	2–3%	33	none	61.0	yes	sp
<i>Trv. winogradskyi</i>	m	no	1.2–1.4	2–3%	33	none	61.0	yes	sp
<i>Thiohalocapsa</i>	no	no	sphere	4–8%	20–30	B <sub>12</sub>	65.9–66.6	yes	ok
<i>Thc. halophila</i>	m	no	1.5–2.5	4–8%	20–30	B <sub>12</sub>	65.9–66.6	yes	ok
<i>Halochromatium</i>	m	no	rod	8–11%	20–30	B <sub>12</sub>	64.6	yes	sp
<i>Hch. salzigens</i>	m	no	2.0–2.5	4–6%	25–35	none	66.1–66.5	yes	sp
<i>Hch. glycolicum</i>	m	no	0.8–1.0	4–6%	25–35	none	66.1–66.5	yes	sp
<i>Isochromatium</i>	m	no	rod	2–3%	25–30	B <sub>12</sub>	62.2–62.8	no	ra
<i>Isc. buderi</i>	m	no	3.5–4.5	2–3%	25–30	B <sub>12</sub>	62.2–62.8	no	ra
<i>Thiospirillum</i>	m	no	Spiral	none	20–25	B <sub>12</sub>	45.5	no	rh, ly
<i>Tsp. jenense</i>	m	no	2.5–4.5	none	20–25	B <sub>12</sub>	45.5	no	rh, ly
<i>Lamprobacter</i>	m	GV	rod	1–2%	25–27	B <sub>12</sub>	64.0	yes	ok
<i>Lpb. modestohalophilus</i>	m	GV	2.0–2.5	1–2%	25–27	B <sub>12</sub>	64.0	yes	ok

abbreviations: m, motile; GV, gas vesicle; o, no information available; ok, okenone; sp, spirilloxanthin; ra, rhodopinal; rh, rhodopin; ly, lycopene; ts, tetrahydro-spirilloxanthin.

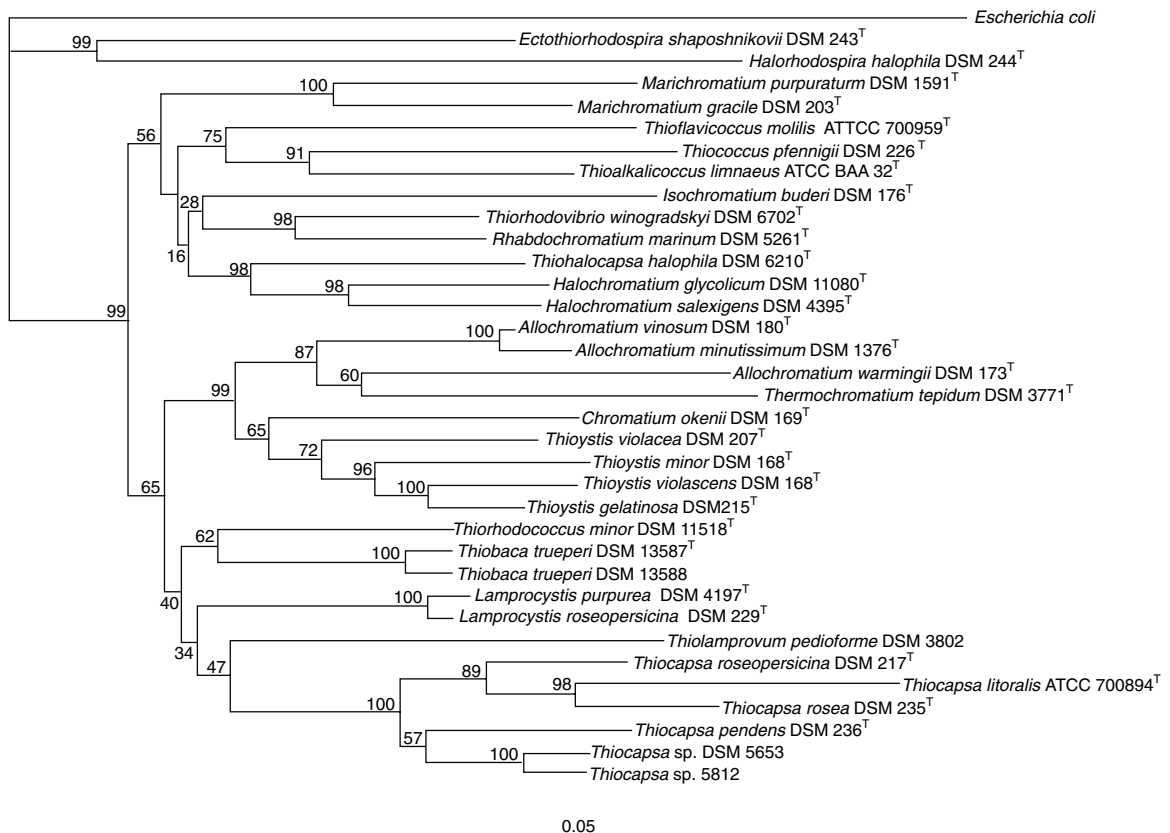


Fig. 1. Phylogenetic tree based on 16S rDNA nucleotide sequences of Chromatiaceae. The 16S rDNA sequences were aligned using the ClustalX program and corrected manually. Calculations were made using maximum likelihood and distance methods. The presented consensus tree was constructed using distance matrices as published previously (Imhoff et al., 1998). Bootstrap analyses with 100 resamplings were performed to obtain estimates for phylogenetic tree topologies. Values at the nodes correspond to bootstrap percentages. The bar corresponds to 5% estimated sequence divergence.

*Thioalkalicoccus*, *Thiorhodovibrio*, *Thiohalocapsa* and *Isochromatium* (Fig. 1). Three genetically related species of this group, which are adapted to the lower range of salt concentrations of brackish and marine habitats (*Thiococcus pfennigii*, *Thioalkalicoccus sibiricus* and *Thioflaviccoccus mobilis*), are clearly distinct from all others by containing bacteriochlorophyll  $\beta$  and by the presence of tubular internal membranes (Bryantseva et al., 2000; Imhoff and Pfennig, 2001b).

Another major branch of the Chromatiaceae is represented mainly by freshwater species (Imhoff et al., 1998). Most of these bacteria do not have a specific requirement for salt, and according to the definitions given by Imhoff (2001a), are considered as freshwater bacteria. They are routinely grown in freshwater media. Several of these bacteria frequently were observed also in marine coastal habitats. Because they are tolerant to salt concentrations of brackish and marine waters and physiologically among the most versatile purple sulfur bac-

teria, they may compete with the marine species of purple sulfur bacteria, in particular at low salt concentrations. Their salt tolerance is sufficient to enable development and competition in numerous coastal habitats. Some species of this group, such as *Thiocapsa litoralis* (Puchkova et al., 2000), even may grow better at minor salt concentrations (1% NaCl) than in its complete absence. This branch includes the genera *Chromatium*, *Allochromatium*, *Thermochromatium* and *Thioystitis* in one group, which are motile forms without gas vesicles. The majority of the genera of a second group (including *Thiocapsa*, *Thiolamprovum*, *Thiobaca* and *Lamprocystis*) is characterized by nonmotile forms with gas vesicles. *Lamprocystis roseopersicina* is one of the rare cases where gas vesicles are formed and the cells are in addition motile by flagella. *Thiobaca trueperi* is a motile rod without gas vesicles (Rees et al., 2002). *Thiocapsa roseopersicina*, one of the best known species of this group, does not form gas vesicles. So far, unpublished sequences of *Thiodictyon* species indicate their association to

the group around *Thiocapsa* species. The 16S rDNA sequences from *Thiospirillum jenense*, *Lamprobacter modestohalophilus* and *Thiopedia rosea* are not yet available.

## Taxonomy

Increasing accumulation of chemotaxonomic and phylogenetic data led to a redefinition of the Chromatiaceae, including the placement of only those purple sulfur bacteria into the family that deposit elemental sulfur inside the cells (Imhoff, 1984b). This definition of Chromatiaceae includes phototrophic purple sulfur bacteria capable of depositing globules of elemental sulfur microscopically visible inside the cells and agrees with Molisch's definition of the "Thiorhodaceae" (Molisch 1907). It is in line with their phylogenetic separation from other phototrophic purple sulfur bacteria, the Ectothiorhodospiraceae (Fowler et al., 1984; Imhoff and Söling, 1996; Imhoff et al., 1998). This definition is, however, in contrast to that of Bavendamm (1924) who included all phototrophic purple bacteria that use sulfide as sole photosynthetic electron donor, and during oxidation, form globules of elemental sulfur, either inside or outside the cells (Pfennig and Trüper, 1971; Pfennig and Trüper, 1974).

### Differentiation of Chromatiaceae and Ectothiorhodospiraceae

Both, Chromatiaceae and Ectothiorhodospiraceae are families of the Chromatiales and also called "phototrophic purple sulfur bacteria." Traditionally, the most important and easily recognized distinguishing property within the Chromatiales is the deposition of elemental sulfur during growth on sulfide. Under anoxic conditions in the light, all Chromatiales are capable of photolithoautotrophic growth and using sulfide or elemental sulfur as electron donor. Elemental sulfur globules accumulate as an intermediate oxidation product of sulfide. Sulfur globules appear microscopically visible either inside the cells (Chromatiaceae) or outside the cells (Ectothiorhodospiraceae). The distinction has traditionally been made by microscopic observation and in most cases is without problems. In one of the recently described species of the Ectothiorhodospiraceae, *Thiorhodospira sibirica*, however, sulfur globules remain attached to the cells and, according to microscopic observations, are located in the cell periphery or the periplasmic space of the cells (Bryantseva et al., 1999). (The localization of sulfur globules in Chromatiaceae is discussed in the subsection Sulfur Metabolism).

Marked differences between representatives of the two families are seen in the 16S rDNA nucleotide sequences, as depicted in a number of characteristic signatures and in their overall sequence distance (Fig. 1).

The two families can also be clearly distinguished by a number of chemotaxonomic properties. Significant differences between Chromatiaceae and Ectothiorhodospiraceae occur in quinone, lipid and fatty acid composition (see Imhoff and Bias-Imhoff, 1995). Characteristic glucolipids are present in Chromatiaceae species, but absent from Ectothiorhodospiraceae (Imhoff et al., 1982). While C-16 fatty acids (in particular C-16:1) are the major components in Chromatiaceae, C-18 fatty acids (in particular C-18:1) are dominant in Ectothiorhodospiraceae, and C-16:1 is only a minor component in this latter group. In addition, the lipopolysaccharides are significantly different in members of the two families (Weckesser et al., 1979; Weckesser et al., 1995). The lipid A of all investigated Chromatiaceae (*Allochromatium vinosum*, *Thermochromatium tepidum*, *Thiocystis violacea*, *Thiocapsa roseopersicina* and *Thiococcus pfennigii*) is characterized by a phosphate-free backbone with D-glucosamine as the only amino sugar, which has terminally attached D-mannose and amide-bound 3-OH-C-14:0. In the lipid A of all tested Ectothiorhodospiraceae (*Ectothiorhodospira vacuolata*, *Ect. shaposhnikovii*, *Ect. haloalkaliphila* and *Hlr. halophila*), phosphate is present, 2,3-diamino-2,3-dideoxy-D-glucose is the major amino sugar (D-glucosamine is also present), D-mannose is lacking (D-galacturonic acid and D-glucuronic acid are present instead), and quite remarkably, 3-OH-C-10:0 is present as an amide-bound fatty acid (Zahr et al., 1992; Weckesser et al., 1995). These distinctive properties of the lipid A appear to be characteristic features of the two families.

### Genera and Species of Chromatiaceae

In the traditional classification, morphological properties such as cell form and size, motility, presence of gas vesicles, and formation of cell aggregates were properties of major relevance. Because this classification was not in congruence with the phylogenetic relationships within the Chromatiaceae species, a reclassification was proposed on the basis of 16S rDNA sequence similarities and with support of selected phenotypic properties (Guyoneaud et al., 1998; Imhoff et al., 1998). New species and genera of Chromatiaceae described since the last edition of this handbook are *Thiorhodovibrio winogradskyi* (Overmann et al., 1992b), *Rhabdochromatium marinum* (Dilling et al., 1995), *Halochromatium glycolicum* (Caumette et al., 1997), *Thiorhodo-*

*coccus minor* (Guyoneaud et al., 1997), *Thioalkalicoccus limnaeus* (Bryantseva et al., 2000), *Thiocapsa litoralis* (Puchkova et al., 2000), *Thioflaviccoccus mobilis* (Imhoff and Pfennig, 2001b) and *Thiobaca trueperi* (Rees et al., 2002). The species of the Chromatiaceae and some selected characteristic properties are shown in Table 1.

## Ecology of Chromatiaceae

Purple sulfur bacteria are widely distributed in nature and are found in all kinds of stagnant water bodies. Ecological niches are those anoxic parts of waters and sediments that receive light of sufficient quantity and quality to allow phototrophic development. The colored blooms often formed by purple sulfur bacteria, already can be seen with the naked eye and naturally have attracted many scientists. In addition to these macroscopically visible blooms, purple sulfur bacteria develop at a level not visible to the naked eye, as is indicated by positive enrichment cultures that were often obtained from sediments or water samples containing hydrogen sulfide but not showing visible accumulations of colored bacteria (e.g., Bavendamm, 1924; Pfennig, 1967; Imhoff, 1988b; Imhoff, 2001a).

Winogradsky (1888) gave a comprehensive description of the different morphological types of purple sulfur bacteria that he observed in samples from nature and raw cultures. Later investigators used Winogradsky's descriptions for identification of purple sulfur bacteria in visible enrichments and natural habitats. In his monograph on the colorless and purple sulfur bacteria, Bavendamm (1924) gave a first detailed account on their ecology, with his own observations and a review of the older literature. A number of more recent reviews consider ecological aspects and the distribution of purple sulfur bacteria in nature (Kondratieva, 1965; Pfennig, 1967; Pfennig, 1989a; Gorlenko et al., 1983; Van Gemerden and Beeftink, 1983; Lindholm, 1987; Imhoff, 1988b; Imhoff, 1992; Imhoff, 2001a; Madigan, 1988; Van Gemerden and Mas, 1995).

The most important properties that determine the development and distribution of phototrophic bacteria in nature are the availability of light, the light intensity, and the concentrations of sulfide and oxygen, which form counter-current gradients in most of the natural habitats of purple sulfur bacteria where sulfide is produced by biological sulfate reduction. The depth at which phototrophic sulfur bacteria develop is largely restricted to the concomitant presence of light and sulfide. Some of the microbial activities that determine slope and position of these gradients, such as oxygen production by algae and

cyanobacteria and sulfide oxidation by phototrophic bacteria, show diurnal, light-dependent fluctuations. Others, such as sulfide production by sulfate-reducing bacteria and oxygen consumption by respiratory bacteria, do not. Because of these processes, the sulfide horizon rises at night and falls again during the day. The motile phototrophic purple bacteria are able to follow the moving sulfide horizon. Under favorable conditions separate layers of green sulfur bacteria are formed underneath layers of phototrophic purple bacteria and of algae and cyanobacteria. Frequently, however, mixed communities of purple and green sulfur bacteria are observed, and purple nonsulfur bacteria regularly accompany the mass development of phototrophic sulfur bacteria.

## The Role of Hydrogen Sulfide

The ability of phototrophic sulfur bacteria to oxidize sulfide and other reduced sulfur compounds under anoxic conditions is one of their most characteristic and ecologically important properties. One of the possible final stages of anaerobic decomposition of organic matter is performed by sulfate-reducing bacteria. Whenever the activity of sulfate-reducing bacteria in a sediment is sufficient to raise the sulfide horizon into the photic zone, development of phototrophic sulfur bacteria is possible. Concentrations of sulfide and elemental sulfur and the relations of the different phototrophic bacteria to these compounds are significant factors in determining patterns of natural dominance and successful competition. Of particular importance are the bacterial affinities for these two sulfur compounds, their oxidation rates, the ability to utilize external elemental sulfur, and the ability to store elemental sulfur inside or outside the cells (Van Gemerden, 1974). The intracellular storage of elemental sulfur by Chromatiaceae gives these bacteria a clear advantage over those bacteria which store elemental sulfur outside the cells (green sulfur bacteria and purple nonsulfur bacteria). All elemental sulfur formed by Chromatiaceae is inaccessible to other bacteria, while the extracellularly formed elemental sulfur, e.g., by green sulfur bacteria is also available to Chromatiaceae. Therefore, intracellular stored elemental sulfur is of inestimable value for these bacteria: 1) under conditions of external sulfide depletion in the light, it serves as a reservoir of photosynthetic electron donors; 2) under dark conditions and in the presence of oxygen, it may support endogenous respiration (Breuker, 1964; Kämpf and Pfennig, 1986); and 3) under anoxic conditions in the dark, it may serve as an electron acceptor during endogenous fermentation of

stored carbohydrates (Hendley, 1955; Van Gernerden, 1968a; Van Gernerden, 1968b).

### The Relations to Oxygen

Although Chromatiaceae are basically anaerobic bacteria and photosynthetic metabolism depends on light and oxygen-deficient conditions, some of the species are considerably tolerant to oxygen and flexible in their metabolism. Many purple sulfur bacteria are able to make use of the oxygen and perform respiratory energy transformations (Kondratieva et al., 1975; Kämpf and Pfennig, 1980).

At least two strategies enable growth, reproduction and successful competition of Chromatiaceae in the overall oxic environment, and are ecologically important: 1) true adaptation by metabolic flexibility in energy conservation, i.e., the ability to readily use both photosynthetic and respiratory mechanisms and 2) the development in and effective use of anoxic niches in an apparent oxic environment.

The first strategy enables the facultative respiring phototrophic bacteria to develop under diurnal oscillating conditions as part-time phototrophs and part-time chemotrophs at the oxic/anoxic chemocline in natural gradient systems. In particular, *Thiocapsa roseopersicina* is of high metabolic flexibility and this species is well equipped to take advantage of changing conditions from oxic/dark to anoxic/light conditions, and even simultaneously drives respiration and photosynthesis (De Wit and van Gernerden, 1990a; De Wit and van Gernerden, 1990b; Schaub and van Gernerden, 1994). Respiratory capabilities not only are of advantage in environments with steep chemical and physical gradients (where during diurnal cycles, changes of the light regime and of oxic to anoxic conditions occur), but also in situations of prolonged darkness where only respiration is possible. *Thiocapsa roseopersicina* also is a good candidate to grow under chemolithotrophic conditions during prolonged dark periods (see Imhoff, 2001a).

An interesting example of the second strategy is the strictly phototrophic *Marichromatium purpuratum*, which has adapted to anoxic niches in the bulk oxic environment and even succeeded in niches in the open ocean. Originally, it was isolated from a strictly oxygen-demanding marine sponge, which contained massive cell densities of this species (Imhoff and Trüper, 1976; Imhoff and Trüper, 1980). Later, a *Didemnum* species revealed similar mass accumulations of this species (see Imhoff, 1992), and more recently, *Marichromatium purpuratum* was isolated from the stomach of marine copepods that have a translucent chitin mantle and thrive in oxic ocean waters (Proctor, 1997).

### The Role of Light

Not only the quantity, but also the quality of light is of major importance for the development of phototrophic bacteria, and owing to the different pigment composition of various phototrophic bacteria, light is also a selective environmental factor. The light quality required by a phototrophic bacterium is reflected in the absorption spectra of the photosynthetic pigment-protein complexes and is a characteristic property for a particular species or group of phototrophic bacteria. Most of the phototrophic purple bacteria have bacteriochlorophyll  $\alpha$ , with long-wavelength absorption maxima between 800 and 900 nm. Only a few species have bacteriochlorophyll  $\beta$ , with absorption maxima at 980–1035 nm.

Light absorption in water masses and sediments follows completely different scenarios (see Imhoff, 1992 and Pfennig, 1989a). In water, the potential light penetration is many meters, while in sediments because of the strong light absorption (Taylor, 1964), the development of phototrophic bacteria is restricted to the uppermost few millimeters. Therefore, the availability of light severely limits the development of phototrophic bacteria in sediments and often they form thin, colored layers below a surface layer of cyanobacteria (Pfennig, 1989a; Imhoff, 1992). Bacteriochlorophyll absorption is of major importance in sediments, particularly sandy sediments where infrared radiation deeply penetrates (Hoffmann, 1949) and favors the growth of bacteria that use photosynthetic pigments (such as bacteriochlorophyll  $\beta$ ) with absorption maxima in the far infrared. Indeed, bacteria with this pigment (e.g., *Thiococcus pfennigii*) are particularly well adapted to sediments not permanently covered by water or covered only by a thin layer such as occurs in many coastal habitats. These bacteria are common to shallow coastal habitats and most of them have been isolated from such locations, but they are rarely encountered in deeper water bodies.

In deeper layers of water, the use of bacteriochlorophylls for light harvesting is limited by the significant absorption of infrared radiation (particularly above 800 nm) by water. Therefore, the role of carotenoids (absorption maxima at 450–550 nm) in light harvesting increases with the water depth. The purple bacteria, in particular those with okenone as carotenoid, have an advantage in deeper water layers because of the efficient light absorption of this pigment.

### Habitats of Chromatiaceae

In addition to the important and selective environmental factors described above (anoxic con-

ditions, and presence of hydrogen sulfide and light), physical and chemical properties such as temperature and salt concentration are important environmental factors relevant for the natural distribution of these bacteria. Most frequently, habitats are those of moderate temperatures, but Chromatiaceae also have been found in thermal springs (Madigan, 1986), and even evidence for their presence in sea ice has been obtained (Petri and Imhoff, 2001). Various species of the Chromatiaceae specifically inhabit different types of freshwater, marine and hypersaline habitats. Because of the striking difference between freshwater and saline habitats, both types of habitats of Chromatiaceae will be discussed separately.

It should be noted that in many investigations tentative identification of purple sulfur bacteria in natural samples was achieved on the basis of microscopic morphological characteristics. Because these properties do not allow species to be identified, assignment of bacteria from natural samples to species and genera is quite problematic, unless isolates are obtained and characterized. In the following, the designations given by the authors of the cited references are used; if the species mentioned has been renamed, the new name is given.

### Freshwater Habitats

Visible accumulations of phototrophic sulfur bacteria occur temporarily in the anaerobic parts of all kinds of freshwater habitats (lakes, shallow ditches, ponds, small stagnant water bodies in forests and botanical gardens, and mountain lakes). In such habitats, development usually proceeds during summer or fall, when abundant hydrogen sulfide is formed by sulfate-reducing bacteria from decaying plant material or from organic pollution. Pink to purple-red blooms of Chromatiaceae are often detected within the water itself or in the form of more-or-less profuse patches that cover the upper or lower side of dead leaves or the mud surface. Early observations on such habitats were already made in the late 19<sup>th</sup> century (Ehrenberg, 1838; Lankester, 1873; Cohn, 1875; Kützing, 1883; Winogradsky, 1888; Miyoshi, 1897), but such observations were much more numerous in the 20<sup>th</sup> century (see below).

The largest and most significant freshwater environments of the phototrophic purple sulfur bacteria are lakes. These habitats are more constant over longer periods of time than others and therefore support more stable phototrophic communities. The blooms of purple (and green) sulfur bacteria usually occur several meters deep at the chemocline and are not visible at the water surface. Depending on the hydrographic conditions, major types of lakes can be differentiated

(Biebl and Pfennig, 1979; Pfennig and Trüper, 1992).

**MEROMICTIC LAKES** Permanently stratified meromictic lakes have an anaerobic, sulfide-containing hypolimnion which often consists of saline water. In these lakes, phototrophic bacteria can thrive at a more or less fixed depth over longer periods. Major fluctuations in the population density occur in response to seasonal differences in the intensity of sunlight and temperature and the associated consequences, including concentrations of sulfide. Examples of meromictic lakes with well developed blooms of purple red layers are the Ritomsee (Switzerland; Duggeli, 1924), the well-studied Lake Belovod (Russia; Kusnetzov, 1970; Sorokin, 1970) and Lake Cadagno (Switzerland; Bosshard et al., 2000) with blooms containing *Chromatium okenii*. Mixed populations of purple sulfur bacteria and green sulfur bacteria were found, e.g., in Suigetsu Lake and Kisaratsu Reservoir (Japan; Takahashi and Ichimura, 1968).

**HOLOMICTIC LAKES** In holomictic lakes that undergo mixing in spring and fall, the anaerobic and sulfide-containing hypolimnion reaches the highest level and hence the strongest illumination condition during summer stratification. Blooms of phototrophic bacteria develop in the uppermost part of the hypolimnion and form colored water layers or plates. In some lakes, this layer is dominated by green sulfur bacteria, in others, green and purple bacteria occur either in mixed populations (e.g., of *Ancalochloris*, *Pelochromatium* and *Lamprocystis*) as in the Pluss-See (northern Germany; Anagnostides and Overbeck, 1966) or develop in different, separated layers, as in Wintergreen Lake (United States; Caldwell and Tiedje, 1975). In this lake, green sulfur bacteria containing gas vesicles thrive below layers with *Thiopedia* and *Thiocystis* species. The Lunzer Obersee (Austria; Ruttner, 1962), the Rotsee (Switzerland; Schegg, 1971) and Lake Vechten (Netherlands; Steenbergen and Korthals, 1982) are examples of holomictic lakes in which populations of *Chromatium okenii*, *Thiopedia rosea* and *Lamprocystis roseopersicina* were reported as the dominant species occurring in purple-red layers. In the Banyoles karstic area of northeastern Spain, a number of small lakes with mass developments of purple sulfur bacteria exist (Guerrero et al., 1987) of which Lake Cisó with blooms of *Chromatium minus* has been studied in most detail (Van Gemerden et al., 1985; Gasol et al., 1991; Pedros-Alio and Guerrero, 1991).

**SHALLOW WATER LAKES** In more shallow lakes with a maximum depth of 15 m, in which the

anaerobic, sulfide-containing zone is primarily confined to the mud sediment and does not extend significantly into the water layers, mass developments of purple sulfur bacteria are more or less restricted to the sediments. Examples of this type of lake are the Kolksee and Edebergsee (northern Germany) with blooms of *Thiopedia* and *Pelochromatium* (Utermöhl, 1925), the Lunzer Mittersee (Austria) with blooms of *Chromatium okenii* and *Lamprocystis* (Ruttner, 1962), Mulizne Lake (Poland) with a *Thiopedia* bloom (Czeczuga, 1968), the monomictic Zaca Lake (California) with a *Thiopedia* bloom (Folt et al., 1989) and Haruna Lake (Japan) with a *Chromatium* bloom (Takahashi and Ichimura, 1968).

**SULFUR SPRINGS** Sulfur springs create aquatic habitats with relatively constant sulfide supply (Winogradsky, 1888), and if exposed to the light, may be suitable habitats for phototrophic sulfur bacteria. Visible mass accumulations of green and purple sulfur bacteria were observed in the effluents of sulfur springs in Poland which contained 40–100 mg of H<sub>2</sub>S/liter (Szafer, 1910; Strzeszewski, 1913). Green sulfur bacteria occurred as the predominant forms at higher sulfide concentrations, followed downstream by different species of purple sulfur bacteria. Purple sulfur bacteria also were found in warm sulfur springs (35–44°C) in Japan (Miyoshi, 1897). While growth temperatures up to 43°C are not uncommon for *Allochromatium vinosum* strains, the optimum growth temperature of 48–50°C with *Thermochromatium tepidum* is exceptional. This moderately thermophilic purple sulfur bacterium was isolated from the Stygian Springs of Yellowstone Park (Madigan, 1986).

**WASTE WATER PONDS** Anaerobic stabilization ponds of wastewater treatment plants also are habitats of purple sulfur bacteria. In general, conditions for the development of purple sulfur bacteria in waste-treatment lagoons are created when wastewater with a high organic load is treated in shallow ponds (1–2 m in depth) which favor the development of anoxic conditions and the formation of sulfide by sulfate-reducing bacteria (Holm and Vennes, 1970; Gloyna, 1971; Sletten and Singer, 1971). In such ponds, blooms of purple sulfur bacteria are mostly visible in spring and fall, while during summer, the ponds often turn green by unicellular algae. The most common purple sulfur bacteria in waste-treatment systems are *Thiocapsa roseopersicina*, *Thiocapsa rosea*, *Thiocapsa pendens* and *Thiolamproyum pedioforme* (Eichler and Pfennig, 1986). In addition, *Thiocystis violacea* and *Allochromatium* species are often present in small numbers.

## Marine Habitats

The shorelines of the oceans with their numerous ecological niches can be considered as the most important places for the development of Chromatiaceae in the marine environment, and the most abundant and conspicuous developments of phototrophic bacteria can be found here. Conditions that favor the development of purple sulfur bacteria are found wherever quiet water is present, e.g., in small splash water ponds along rocky shores, in seawater pools and small puddles of salt marshes, in closed bays and large lagoons, in sediments of sandy beaches, salt marshes and tidal flats, and in muddy masses of decaying algae or seaweeds. In these places, bacterial sulfate reduction is facilitated by the degradation of decaying organic materials and the presence of abundant sulfate (Trüper, 1980). Such habitats have also been named “beach sulfureta” (Suckow, 1966) because they house a complete sulfur cycle including bacterial sulfate reduction and sulfide oxidation by phototrophic bacteria and by chemotrophic sulfur bacteria. Chromatiaceae are the most obvious and numerous phototrophic bacteria in these habitats. Since Warming (1875) described mass developments of purple sulfur bacteria on the coasts of Denmark, these bacteria have been found and studied along the ocean coasts all over the world (for reviews, see Pfennig, 1967; Pfennig, 1989a; Trüper, 1980; Pfennig and Trüper, 1981; Imhoff, 1988b; Imhoff, 1992; Imhoff, 2001a; Van Gemerden and Mas, 1995).

It is supposed that the metabolic versatility is an important property to explain their widespread occurrence in the highly dynamic and unstable marine coastal habitats (Imhoff, 2001a). Great metabolic versatility is of selective advantage at these habitats, and the most versatile phototrophic purple bacteria frequently are among the dominant species here (Molisch, 1907; Bavendamm, 1924; Trüper, 1970; Imhoff, 1992; Imhoff, 2001a). This versatility is reflected 1) in the ability to use different possibilities of energy conservation and different photosynthetic electron donors, in particular sulfide and thiosulfate, 2) in the high potential of photoheterotrophic growth together with the ability to assimilate sulfate as sole sulfur source, and 3) in the ability to grow chemotrophically under oxic conditions in the dark, either autotrophically or heterotrophically. In particular *Allochromatium vinosum*, *Marichromatium gracile*, *Thiocystis violacea* and *Thiocapsa roseopersicina* have regularly been observed and isolated from marine coastal habitats. Other species frequently encountered are *Allochromatium warmingii*, *Thiocystis violas-cens*, *Allochromatium minutissimum*, *Thiocystis minor*, *Thiocapsa rosea*, *Marichromatium purpu-*



*ratum* and *Isochromatium buderi* (Trüper, 1970; Trüper, 1980; Imhoff, 1988b; Imhoff, 1992; Imhoff, 2001a; Pfennig, 1989a).

**SEDIMENTS AT MARINE SHORELINES** Owing to their dependence on the process of bacterial sulfate reduction, which is by and large restricted to the sediments, phototrophic sulfur bacteria are more or less confined to the sediments or to sediment-associated waters that receive sulfide from these sediments. Whenever the production of sulfide is not sufficient to penetrate into the water, development of these bacteria is restricted to the sediment. Under such conditions, they often form thin colored layers within the top millimeters of the sediments. Sandy beaches with such colored layers were called the "Farbstreifensandwatt" (Schulz, 1937; Schulz and Meyer, 1939; Hoffmann, 1942; Hauser and Michaelis, 1975). Microbial mats of phototrophic microorganisms, including layers of purple sulfur bacteria, also are common to sheltered areas of the Waddensea and of salt marshes.

An outstanding example is found in the microbial mats of the Great Sippewissett Salt Marsh (Cape Cod, MA, United States), where laminated microbial mats of unusual thickness regularly occur during summer and one very well developed mat was described by Nicholson et al. (1987). In these sandy intertidal sediments, the mats were about 10 mm thick and comprised four to five distinctly colored layers. Phototrophic purple sulfur bacteria of the central pink layer and the directly underlying peach-colored layer were identified. The dominant bacteria of the pink layer were considered to be *Thiocapsa roseopersicina* and those of the peach layer were recognized as *Thiococcus pfennigii* on the basis of the presence of bacteriochlorophyll  $\beta$  and the bundles of tubular intracellular membranes in the coccoid cells. A second bacterium with bacteriochlorophyll  $\beta$  was isolated from this layer. It resembles *Thiococcus pfennigii* in many properties but is a motile coccus and was described as a new species, *Thioflavococcus mobilis* (Imhoff and Pfennig, 2001b). Also two new species of purple nonsulfur bacteria have been described recently from this location. In the peach-colored layer of the mats, small spirilloid bacteria were detected by scanning electron microscopy (about 1% of total cells), which were isolated and found to contain bacteriochlorophyll  $\beta$  and to exhibit a number of unusual characteristics. This bacterium was described as the new species *Rhodospira trueperi* (Pfennig et al., 1997). Another purple nonsulfur bacterium, *Roseospirillum parvum*, also was isolated from this habitat (Glaeser and Overmann, 1999). In addition, a greater number of isolates of purple and green sulfur bacteria

are known to originate from this salt marsh (Trüper, 1970).

**COASTAL WATERS, LAGOONS, AND FJORDS** There are a number of reports on the development of massive blooms of phototrophic bacteria, sometimes called "red waters" in shallow coastal waters such as lagoons but also in saline lakes and anoxic fjords. Examples are Lake Faro in Sicily (Genovese, 1963; Trüper and Genovese, 1968); the Mar Piccolo near Trento (Cerruti, 1938); Veliko and Male Jezero on the Dalmatian island of Mljet (Cviic, 1955; Cviic, 1960); the Bay of Tunis (Heldt, 1952; Stirn, 1971) and Bietri Bay of the Ebric Lagoon, Ivory Coast (Caumette, 1984); the Etang du Prevost near Montpellier, France (Caumette, 1986); lagoons at Messolonghi and Aitolikon in Greece (Hatzikakidis, 1952; Hatzikakidis, 1953); the Solar Lake on the Sinai Peninsula (Cohen et al., 1977); Lake Mogilnoye on the Arctic island of Kildin (B. L. Isachenko [1914], "Studies of bacteria of the Arctic Ocean," cited in Gorlenko et al., 1978; Gorlenko et al., 1978) and others.

**THE BLACK SEA** In contrast to estuarine or near-shore marine habitats, the oxygenated waters of the open ocean have been found to be devoid of phototrophic sulfur bacteria. A special case of a stratified marine habitat is the Black Sea, a large, meromictic enclosed basin that is not part of the open ocean. Although its anoxic layer did not reach the photic zone and the development of anoxygenic phototrophic bacteria seemed to be impossible, their presence in the Black Sea was first demonstrated by Kriss and Rukina (1953). Later, several Chromatiaceae species (*Thiocapsa roseopersicina* and *Allochromatium warmingii*), but also green sulfur bacteria (*Chlorobium phaeobacteroides*) were obtained in enrichment and pure cultures from anoxic dark Black Sea bottom sediments at 600 and 2240 m depth (Hashwa and Trüper, 1978). These authors assumed survival of the phototrophic bacteria that may be washed away from their estuarine environment into the dark layers of the Black Sea, but excluded active growth under the anoxic dark conditions in the deep sediments. The situation was much different approximately 10 years later, when the chemocline reached horizons (68 to 90 m) receiving light of minor intensities. At that time large amounts of bacteriochlorophyll, indicative of brown-colored green sulfur bacteria, were detected (Repeta et al., 1989; Jørgensen et al., 1991; Overmann et al., 1992a). Apparently light intensities at the depth of the chemocline still were insufficient to support the development of purple sulfur bacteria.

**SEA ICE** Sea ice is generally regarded to be oxic but not anticipated to have anoxic niches and

therefore not to be a habitat of anoxygenic phototrophic bacteria. The possible existence of anoxic niches within the complex system of small brine channels within sea ice was concluded from the presence of various types of anaerobic and potentially anaerobic bacteria in the interior of sea ice from the Baltic Sea (Petri and Imhoff, 2001). As revealed by 16S rDNA analyses, a Chromatiaceae species is a major component within a distinct layer of this sea ice. These findings point out that sea ice may contain potential niches for anoxygenic phototrophic bacteria. Again, oxygen tolerance and respiratory capabilities are considered to be excellent prerequisites for them to live in such a habitat (Petri and Imhoff, 2001).

**SALT AND SODA LAKES** Though many habitats of phototrophic bacteria in the coastal zone contain brackish waters, others are more concentrated than seawater. Shallow waters in splash water ponds, coastal lagoons, closed basins and the like, which receive intensive illumination from the sun are subject to evaporation. As a consequence, inorganic salts and organic matter accumulate and chances for the occurrence of anoxic conditions and the development of anoxygenic phototrophic bacteria increase. These waters have higher fluctuations in salt concentrations and quite often the salinities are higher than in seawater. Many isolates from such places are tolerant to a wide range of salt concentrations and some require or tolerate salt concentrations above seawater salinity (Imhoff, 1988b; Imhoff, 1992; Imhoff, 2001a). Particularly high fluctuation occurs in the so-called "splash water ponds" along rocky coasts and these have been found to contain phototrophic sulfur bacteria. Such habitats were studied in Japan (Taga, 1967), Helgoland (Germany), and Yugoslavia (Imhoff, 1988b).

Thalassohaline waters occur as natural evaporation pools of marine waters or as man-made evaporation ponds of marine salterns. A different type of hypersaline environment is found in various athalassohaline waters of inland salt water lakes. A few prominent examples of such lakes are the Great Salt Lake (Utah), the Dead Sea (Israel), and the soda lakes of the Wadi Natrun in Egypt. These habitats have largely different ionic compositions (Imhoff et al., 1979). Evidence for the presence of ecological niches for halophilic phototrophic purple bacteria and their occurrence is available for most of these lakes, and in some of them, the development in visible masses has been described (see Imhoff, 1988b; Imhoff, 1992; Imhoff, 2001a). Numerous isolates have been obtained and a number of new species of phototrophic purple sulfur bacteria have been described from marine salterns (Ca-

umette, 1993; Caumette et al., 1988; Caumette, 1991; Caumette, 1994), alkaline soda lakes in the Egyptian Wadi Natrun (Jannasch, 1957; Imhoff and Trüper, 1977; Imhoff and Trüper, 1981; Imhoff et al., 1979), Russian soda lakes in Siberia and Mongolia (Bryantseva et al., 1999; Bryantseva et al., 2000), and the Solar Lake (Cohen et al., 1977; Imhoff, 1983; Caumette et al., 1997).

## Selective Enrichment of Chromatiaceae

Ever since the first experimental studies on purple sulfur bacteria by Winogradsky (1888), it has been customary to grow these bacteria in the laboratory in raw enrichment cultures. These well-known Winogradsky columns are set up in tall glass cylinders, e.g., with plant residues, CaSO<sub>4</sub>, anaerobic mud and water of a natural habitat. Traditionally, they are incubated in dim light. Variations of this column technique are discussed by Pfennig (1965) and van Niel (1971). The preparation and development, as well as the advantages and limitations, of the different types of such long-lasting raw cultures are well described (Winogradsky, 1888; Buder, 1915; Schrammeck, 1934; Schlegel and Pfennig, 1961; Pfennig, 1965; van Niel, 1971). Most purple sulfur bacteria that thrive in Winogradsky columns also can be grown and isolated by direct methods in synthetic media (see the section Isolation in this Chapter).

Alternatively, phototrophic sulfur bacteria may be selectively enriched from most natural habitats in suitable media under anoxic conditions and in the light. Medium 1 (see below), eventually with modifications and different supplements, is a good choice for the selective enrichment of Chromatiaceae from freshwater and marine habitats. For this purpose, pH adjustment to 7.2 and 7.4 is recommended. For the successful enrichment of purple sulfur bacteria, it is important to realize that many species quite specifically occur in characteristic ecological niches. The species composition of the inoculum is, therefore, of primary importance for the outcome of enrichment experiments.

Of general importance for the selectivity of enrichment cultures for Chromatiaceae (and other anoxygenic phototrophic bacteria) are the culture media and the incubation conditions, in particular, the mineral salts composition and salinity, the concentration of nutrients, the presence of vitamins, as well as pH, temperature, light intensity and light regime. The choice of the carbon source is not critical for the success of such enrichment cultures because fermentative processes in natural enrichments usually result in

the formation of acetate and/or other acids (propionate, butyrate and lactate), which are good substrates for the majority of the purple sulfur bacteria.

### Sulfide Concentration

A number of species are inhibited by higher sulfide concentrations. Therefore, the sulfide concentrations should be kept as low as 1–2 mM, so that the cultivation of sensitive forms is not excluded. High population densities can be achieved only by repeated sulfide “feeding” (i.e., addition of a neutralized sulfide solution). *Thiopedia rosea* is exceptional in being inhibited by sulfide concentrations already above 0.6 mM, and the addition of sodium dithionite (50 mg for 1 liter) is required for cultivation of this bacterium (Pfennig and Trüper, 1992).

### Salinity and Mineral Salts Composition

This is of special importance, if samples from marine and hypersaline environments are investigated. The salinity of the enrichment culture is usually adjusted according to the salinity of the inoculum. For marine isolates, it is sufficient to raise the NaCl concentration to 2–3%. Some isolates also require increased concentrations of magnesium (e.g., 0.3% MgSO<sub>4</sub> · 7H<sub>2</sub>O) and sometimes also of calcium (Biebl and Pfennig, 1978). Notably, the salinity of a natural habitat may not always be optimal for a particular isolate from the habitat. Therefore, use of enrichment media of different salinities may be useful for the isolation of a variety of species from the same sample.

### Incubation Temperature

Temperature is important for the enrichment culture of Chromatiaceae. As a rule, at high incubation temperatures (28–35°C), a wide variety of different species are outgrown by single, fast-growing species. At low temperatures (15–22°C), the enrichment cultures develop more slowly and a larger number of different species may be present simultaneously. Elevated temperatures above 40°C are highly selective for moderately thermophilic species such as *Thermochromatium tepidum* (Madigan, 1986).

### Illumination and Light Intensity

The various species of the purple sulfur bacteria differ with respect to the selective advantage they exhibit under different kinds of illumination. These differences can be exploited for the selective enrichment of certain groups of species (Pfennig, 1965; Pfennig, 1967). Two different illumination schemes with incandescent light are recommended here.

If continuous illumination at high light intensities of 1,000–2,000 lux, and an incubation temperature of about 30°C is used, the small and fast-growing Chromatiaceae can be expected to enrich, e.g., *Thiocapsa roseopersicina*, *Allochromatium vinosum*, *Allochromatium minutissimum*, *Thiocystis minor*, *Thiocystis violascens*, *Thiocystis violacea* and *Marichromatium gracile*.

Results may be different with intermittent illumination at low light intensities of 50–300 lux and an incubation temperature of about 20°C. Duration of the light and dark phases may be varied from 12 h light and 12 h dark (6 h light and 6 h dark) as proposed by Pfennig (1967) to 4 h light and 8 h dark (van Gernerden, 1974). Depending on the inoculum, the flagellated, large cell Chromatiaceae and those containing gas vesicles can be expected to enrich, e.g., *Thiospirillum jenense*, *Chromatium okenii*, *Chromatium weissei*, *Allochromatium warmingii*, *Isochromatium buderi*, *Thiocystis gelatinosa*, *Lamprocystis roseopersicina*, *Thiodictyon elegans*, *Thiocapsa rosea* and *Thiopedia rosea*. The flagellated forms keep swarming in the whole bottle (Pfennig, 1962) and can be further enriched by carefully pipetting the inoculum for subsequent enrichment cultures from the upper part of the culture bottle. During incubation of the enrichment cultures, at first, the nonmotile forms enrich at the bottom. Later, the cells containing gas vesicles tend to accumulate at the surface under the screw cap. This process can be accelerated by keeping the enrichment for a few days in a refrigerator at 4°C. For further enrichment, the floating cell mass is carefully pipetted from the surface and transferred to fresh medium.

The use of specific light filters can be of value for the enrichment of purple sulfur bacteria. Development of green sulfur bacteria with their long wavelength absorption maxima between 705 and 750 nm can be prevented when the enrichments are illuminated behind an infrared filter that transmits light only above 800 nm. The selective enrichment of bacteria that contain bacteriochlorophyll β, such as *Thiococcus pfennigii* (Eimhjellen et al., 1967; Eimhjellen, 1970) and *Thioflavicoccus mobilis* (Imhoff and Pfennig, 2001b) is achieved with an infrared filter that transmits radiation only above 900–1,000 nm.

## Isolation

The first rational enrichment and isolation procedures for purple and green sulfur bacteria were developed by van Niel (1931). Further attempts to grow in pure culture the large-celled purple sulfur bacteria *Chromatium okenii* and *Thiospirillum jenense* led to the elaboration of a

synthetic medium that contains vitamin B<sub>12</sub> (Pfennig, 1965; Pfennig and Lippert, 1966). An advanced recipe of this medium will be given below (Medium 1). With slight modification, this culture medium allows the isolation and cultivation of most purple (and green) sulfur bacteria that occur in freshwater, estuarine and marine habitats.

### Direct Isolation

As an alternative to enrichments followed by pure culture isolation, pure cultures may be obtained by directly inoculating agar media from natural samples without prior enrichment. This strategy has to be used whenever the analysis of the natural diversity is attempted and information on the natural abundance and distribution of the species in a sample is desired. Media with low selectivity are required for this approach. When the incubation time is adapted to the growth rates of slowly growing strains or of those not well adapted to the given conditions, separation in or on agar allows these cells to grow into small colonies that can be picked up for further transfers.

Deep agar dilution series (see below) should be prepared directly from the sample without prior enrichment in liquid culture. Even in this case, however, the incubation conditions for the agar cultures should closely resemble the conditions used for liquid enrichment cultures of the desired bacteria, i.e., use of low temperatures and low light intensities as indicated above is recommended.

Methods of direct isolation of the phototrophic bacteria from a natural sample use agar dilution series or inoculation of agar plates to separate the cells prior to incubation. For inoculation, a sample of water, mud, sludge, or even soil may be used as a homogeneous suspension in medium or in filter-sterilized water from the habitat. Samples containing less than 10 cells/ml need to be concentrated by centrifugation (agar dilution series) or filtration (agar plates). All methods for direct isolation are suitable for the determination of living cell counts, when known amounts of the sample are used in appropriate dilutions.

### Isolation Procedures

**MEDIA FOR CULTIVATION OF PURPLE SULFUR BACTERIA** The composition and preparation of two different culture media are described below for the cultivation of purple sulfur bacteria. The first (Medium 1) is suitable for almost all purple bacteria presently in laboratory culture, including those species that are most difficult to grow

(e.g., *Thiopedia rosea*, *Thiospirillum jenense*, *Chromatium okenii* and *Thiodictyon elegans*). With minor modifications, this culture medium formulation was published by Pfennig (1965), Pfennig and Lippert (1966), Trüper (1970), Eichler and Pfennig (1988) and Pfennig and Trüper (1992). In addition, a second, very similar culture medium is given (Medium 2), which is easier to prepare and which allows the cultivation of most of the common purple sulfur bacteria (Biebl and Pfennig, 1978).

#### Medium 1 (Eichler and Pfennig, 1988)

The medium is prepared in a 5-liter bottle with four openings at the top. Two openings are connected to tubing: 1) a short, gas inlet tube with a sterile cotton filter and 2) an outlet tube for the medium, which reaches the bottom of the central part of the vessel at one end and has, at the other end, a silicon rubber tube with a pinchcock and a bell for aseptic dispensing of the medium into bottles. The other two openings have gas-tight screw caps. One of these openings is for the addition of sterile solutions and the other can serve as a gas outlet.

##### Solution 1

Distilled water	4,900 ml
KH <sub>2</sub> PO <sub>4</sub>	1.7 g
NH <sub>4</sub> Cl	1.7 g
KCl	1.7 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	2.5 g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	1.25 g

Solution 1 is autoclaved for 45 min at 121°C in the 5-liter bottle, together with a teflon-coated magnetic stir bar. It is cooled to room temperature under an N<sub>2</sub> atmosphere with a positive pressure of 0.05–0.1 atm (a manometer for low pressures is required). The cold medium is saturated with CO<sub>2</sub> by magnetic stirring for 30 min under a CO<sub>2</sub> atmosphere of 0.05–0.1 atm. The sterile solutions 2 through 5 (see below) are then added through one of the screw-cap openings against a stream of either N<sub>2</sub> gas or, better, a mixture of 95% N<sub>2</sub> and 5% CO<sub>2</sub> while the medium is magnetically stirred.

For enrichment cultures or pure cultures from marine or estuarine habitats, 100 g of NaCl are added to solution 1 and the content of MgSO<sub>4</sub> · 7H<sub>2</sub>O is increased to 15 g.

#### Solution 2: Vitamin B<sub>12</sub> Solution

A sterile-filtered stock solution containing 2 mg of vitamin B<sub>12</sub> in 100 ml of distilled water is prepared, and 5 ml are added to Solution 1.

#### Solution 3: Trace Element Solution SL 12

Distilled water	1 liter
Ethylene diamine tetraacetate-Na <sub>2</sub>	3.0 g
FeSO <sub>4</sub> · 7H <sub>2</sub> O	1.1 g
H <sub>3</sub> BO <sub>3</sub>	300 mg
CoCl <sub>2</sub> · 6H <sub>2</sub> O	190 mg
MnCl <sub>2</sub> · 4H <sub>2</sub> O	50 mg
ZnCl <sub>2</sub>	42 mg
NiCl <sub>2</sub> · 6H <sub>2</sub> O	24 mg
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	18 mg
CuCl <sub>2</sub> · 2H <sub>2</sub> O	2 mg

The salts are dissolved in the order given, the pH is adjusted to 2–3 with HCl, the solution is sterilized, and 5 ml are added to solution 1.

**Solution 4: Na-bicarbonate Solution**

A solution of 7.5% Na-bicarbonate is prepared, saturated with CO<sub>2</sub>, autoclaved under a CO<sub>2</sub> atmosphere in a tightly closed bottle, and 100 ml are added to solution 1.

**Solution 5: Sodium Sulfide Solution**

A 10% Na<sub>2</sub>S · 9H<sub>2</sub>O solution is prepared in a screw-cap bottle, and after replacement of the air by N<sub>2</sub>, the bottle is tightly closed and autoclaved. Twenty ml of the sterile solution are added to solution 1.

After combining and carefully mixing solutions 1 through 5, the pH of the medium is adjusted by stirring under an atmosphere of CO<sub>2</sub> (0.5 bar pressure) for approx. 40 min to pH 7.2. The medium is then immediately dispensed aseptically under pressure of N<sub>2</sub> (0.05–0.1 atm) into sterile 100-ml bottles with metal screw caps containing autoclavable rubber seals. A really small, pea-sized air bubble is left in each bottle to meet possible pressure changes. The tightly sealed screw-cap bottles can be stored for several weeks to a month in the dark. During the first 24 h, some trace metal (mainly iron) sulfides of the medium precipitate in the form of fine black particles. No other sediment should arise in the otherwise clear medium.

**Supplement Solutions****Sulfide Solution for Feeding of Batch Cultures**

The amount of Na<sub>2</sub>S · 9H<sub>2</sub>O initially added to Medium 1 (higher initial amounts may be inhibitory for some species) will only produce very limited growth. After the sulfide and sulfur are completely oxidized, the bacteria stop growing and may be damaged by further illumination. To keep the cultures growing and to obtain high cell yields, it is necessary to feed the cultures several times with sterile, partially neutralized sulfide solution. Two different sulfide feeding solutions are prepared as follows:

Dissolve 3.6 g of Na<sub>2</sub>S · 9H<sub>2</sub>O in 100 ml of distilled water (a 0.15M solution) in a 250-ml screw-cap bottle. After replacement of the air by N<sub>2</sub>, the bottle is tightly closed and autoclaved. To prepare the neutralized feeding solution, a measured amount of this solution is added to a sterile Erlenmeyer flask containing a magnetic stir bar. The solution is brought to about pH 7.3 by dropwise addition of sterile 1M H<sub>2</sub>SO<sub>4</sub> on a magnetic stirrer. If too much acid is added, the sulfide solution becomes turbid owing to precipitation of elemental sulfur. The nearly neutralized solution is immediately used for feeding 100-ml bottle cultures. Depending on the population density, 1–2 ml are used for Chromatiaceae. Before the addition, an equivalent amount of culture medium is aseptically removed from the bottle culture.

Alternatively the method of Siefert and Pfennig (1984) offers a ready to be used, neutralized sulfide feeding solution. This solution is prepared in a 500-ml bottle with a small outlet at the bottom, connected by a rubber tube with a pinchcock to a test tube tightly closed by a screw cap (Siefert and Pfennig, 1984). In a volume of 250 ml of distilled water 7.0 g of Na<sub>2</sub>S · 9H<sub>2</sub>O and 2.65 g of Na<sub>2</sub>CO<sub>3</sub> are dissolved and autoclaved under a CO<sub>2</sub> atmosphere. The sterile solution is saturated with CO<sub>2</sub> under pressure

(0.8 bar) until the pH is approx. 7.3. The solution is stored under CO<sub>2</sub> pressure and ready for use. For feeding of cultures, a small volume is pressure-released from the bottle into the test tube and transferred from this tube into the cultures by using a sterile pipette.

**Thiosulfate Solution for Cultivation of Chromatiaceae**

Cultures of purple sulfur bacteria that can use thiosulfate as an electron donor can be supplemented with 0.1% of this compound from a stock solution (dissolve 10 g of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> · 5H<sub>2</sub>O to a final volume of 100 ml of distilled water). This solution is prepared in a 200-ml screw-cap bottle and autoclaved. One ml of this solution is added aseptically to 100 ml of culture medium.

**Acetate Solution for Cultivation of Chromatiaceae**

Growth yields of purple sulfur bacteria can be increased by the addition of acetate as a readily assimilated carbon source. The ammonium and magnesium salts of acetate are used to avoid strong pH changes during growth. Ammonium acetate (2.5 g) and magnesium acetate (2.5 g) are dissolved in 100 ml of distilled water and the solution is autoclaved. Standard application is 1 ml added aseptically to 100 ml of culture medium. The addition of acetate solution to media used for deep agar dilution series of Chromatiaceae is highly recommended. To liquid cultures, acetate should be added only if these are free of competing contaminants such as purple nonsulfur bacteria.

**Medium 2 (Biebl and Pfennig, 1978)****Solution 1**

Distilled water	950 ml
KH <sub>2</sub> PO <sub>4</sub>	1 g
NH <sub>4</sub> Cl	0.5 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.4 g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.05 g

For marine strains, 20 g of NaCl are added to solution 1 and the amount of MgSO<sub>4</sub> · 7H<sub>2</sub>O is increased to 3 g.

The solution is autoclaved in the cotton-plugged 2-liter Erlenmeyer flask with an outlet near the bottom. A silicon rubber tube (about 30 cm long) with a pinchcock and a bell for aseptic distribution of the medium into bottles is connected to the outlet. A magnetic stir bar is put into the flask. When the autoclaved solution 1 is cooled to room temperature, the following sterile solutions 2 through 5 are aseptically added while magnetically stirring the medium.

**Solution 2: Vitamin B<sub>12</sub> Solution**

The solution is prepared as a sterile-filtered stock solution containing 2 mg of vitamin B<sub>12</sub> in 100 ml of distilled water, and 1 ml is added to solution 1.

**Solution 3: Trace Element Solution SL 12**

The composition is as given under Medium 1 (see above). Application is 1 ml per 1 liter of medium.

**Solution 4: Sodium Bicarbonate Solution**

A 5% NaHCO<sub>3</sub> solution is prepared in distilled water, filter-sterilized, and 30 ml are added to solution 1.

**Solution 5: Sodium Sulfide Solution**

A freshly autoclaved 6% solution of Na<sub>2</sub>S · 9H<sub>2</sub>O is prepared in distilled water, and 6 ml are added to medium.

After the mixing of solutions 1 through 5, the pH of the medium is adjusted with sterile 1M H<sub>2</sub>SO<sub>4</sub> or 1M Na<sub>2</sub>CO<sub>3</sub> solution to pH 7.2, and the volume brought to 1 liter with sterile distilled water, if necessary. The medium is then dispensed aseptically into sterile, 50- or 100-ml bottles with metal screw caps containing autoclavable rubber seals. A small air bubble is left in each bottle to meet possible pressure changes.

### Methods for Pure Culture Isolation

Irrespective of the source of the inoculum (e.g., sample from nature, enrichment culture, or suspension of a colony), the deep agar dilution method is the most convenient method for preparing pure cultures of phototrophic sulfur bacteria (Larsen, 1952; Pfennig, 1965; Trüper, 1970; Imhoff 1988a). For the large, motile species that do not easily form colonies in agar media (e.g., *Thiospirillum jenense*) either reduced agar concentrations are applied (0.6%) or Giesberger's "Pasteur pipette" method (Giesberger, 1947) is recommended (see Pfennig and Trüper, 1992).

**PREPARATION OF AGAR DILUTION SERIES** For agar dilution series, selective media are not required and nonselective ones are preferred for a direct isolation without prior enrichment procedure. In a modification of the method of Pfennig (Pfennig, 1965; Trüper, 1970; Imhoff, 1988a), purified agar (thoroughly washed several times with distilled water and at a final concentration of 1.8%) is dissolved in distilled water and distributed in amounts of 3 ml into cotton-plugged test tubes. (For marine samples, 2% NaCl is added to this agar solution.) The agar is liquefied, and while kept in a hot-water bath, it is dispensed in 3-ml portions into standard test tubes, which are then plugged with cotton and autoclaved. For dilution series, the liquid agar is kept at 50°C in a water bath until use. A suitable medium is placed in the same water bath, and 6 ml of the prewarmed medium is added to each test tube. Medium and agar are mixed thoroughly by turning the tubes upside down and back, and kept at 50°C. Eight tubes are sufficient for each dilution series. The first tube is inoculated with 1–3 drops from a natural sample or enrichment culture of phototrophic bacteria; the contents are immediately mixed by inverting the tube once. Of this inoculation, 0.5–1.0 ml are then transferred into a second tube that contains the agar medium, mixed immediately by inverting as with the first tube, and so on. This dilution series is continued over eight steps. After transfer to the next tube, each tube is set into a water bath with tap water to harden the agar. After the agar has hardened, the tubes are sealed with a paraffin mixture (3 parts paraffin oil and 1 part paraffin) to prevent diffu-

sion of oxygen into the agar. Alternatively, anoxic conditions can be maintained by applying an oxygen-free gas phase. In this case, the cotton plugs are replaced by rubber stoppers after hardening of the agar. The air above the agar is replaced by gassing with sterile N<sub>2</sub> and 5% CO<sub>2</sub>, and the tubes are then tightly closed with the rubber stoppers. The agar tubes are kept in the dark for several hours before they are incubated under appropriate conditions. Standard conditions routinely applied are at 20–28°C and at a light intensity of 200–1000 lux.

After cells have grown to visible colonies, the paraffin layer is removed by melting and the tubes are turned upside down on a cotton towel. Individual colonies are isolated from appropriate dilutions that shows well separated pigmented colonies. They are picked with a Pasteur pipette (the tip drawn out to a thin capillary and attached to a rubber tube). The content of the colony is suspended in 0.5 ml of sterile medium in a test tube, the suspension is microscopically checked for purity, and the whole dilution series in deep agar tubes is repeated.

In general, at least three to four such dilution series are necessary to obtain pure cultures. When pure cultures have been obtained, single colonies are inoculated into liquid medium. It is advisable to start with small-sized bottles or screw-cap tubes (10- or 25-ml) and to scale up to the regularly used sizes in following transfers of the grown culture.

**CULTIVATION ON AGAR PLATES IN ANAEROBIC JARS** Purple sulfur bacteria have also been successfully isolated on agar plates. A useful method for cultivating phototrophic sulfur bacteria was introduced by Irgens (1983). It is based upon the release of hydrogen sulfide, ammonia, and acetic acid from decomposing thioacetamide. The agar medium is prepared without any sulfide and poured into Petri dishes. After inoculation, these are placed in anaerobic jars (for instance, the Gas-Pak system of Becton Dickinson, Cockeysville, MD). Before the jars are closed, they are supplemented with a test tube or a small beaker containing 0.05–0.1 g of thioacetamide (depending on the size of the jar and the desired concentration) suspended in 1.0 ml of 0.2N or 0.5N HCl. The hydrogen sulfide is slowly released over a period of at least one week (Irgens, 1983). Also included in the jars are the methylene blue redox indicator and a strip of lead acetate-sulfide indicator. This method may be applied to obtain viable cell counts and was successfully used for the isolation of species of the genera *Allochromatium*, *Lamprocystis*, *Thiocapsa*, *Thiocystis* and *Ectothiorhodospira* with an illumination by a 60-W incandescent light bulb at a distance of 60 cm from the jars (Irgens,

1983). When high numbers of phototrophic bacteria are present in the sample, streaking by conventional methods is appropriate. Samples containing low numbers of phototrophic bacteria can be easily concentrated on membrane filters (e.g., cellulose acetate or cellulose nitrate).

## Identification

Identification of new isolates can be obtained only by detailed studies of physiological and morphological properties together with genetic sequence data. It is not possible to identify phototrophic bacteria only from microscopic observations of natural samples or enrichment cultures. To unambiguously distinguish closely related strains and species of the same genus, often DNA-DNA hybridization studies are required.

Morphological properties obtained by microscopic techniques can provide, however, first information about cell structures (form, size and motility of the cells) and inclusions (sulfur globules, gas vesicles, and storage polymers). The cell morphology of a few representative species using light microscopy is shown in Fig. 2.

Ultrathin sections under the electron microscope reveal the fine structure of the cells, in particular, the type of the internal membrane system. The internal photosynthetic membranes are of vesicular type in most Chromatiaceae, but form tubules in some species (see Fig. 3). They carry the pigment-protein complexes of the photosynthetic apparatus.

The color of cell suspensions and absorption spectra yield preliminary information on the predominant bacteriochlorophylls and on the kind of bacteriochlorophyll-protein complexes. Photosynthetic pigments are bacteriochlorophyll  $\alpha$

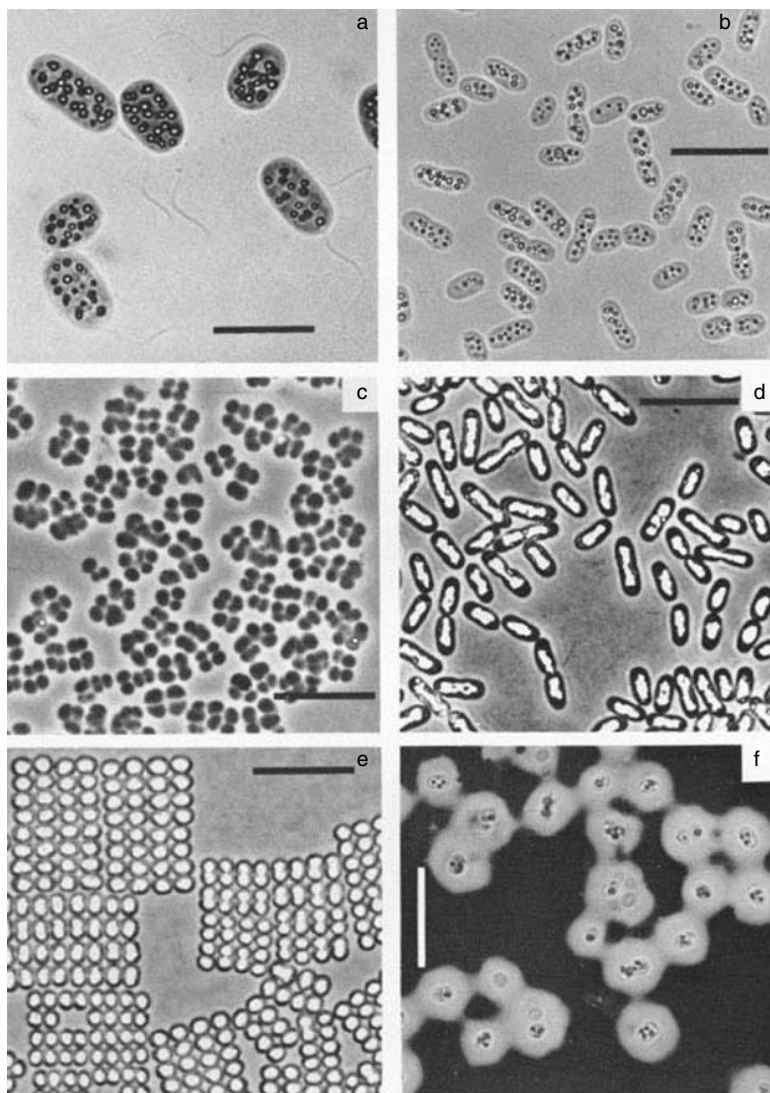


Fig. 2. Morphology of the Chromatiaceae. (a) *Chromatium okenii* (light-field micrograph); (b) *Allochromatium vinosum* (light-field micrograph); (c) *Thiocapsa roseopersicina* (phase contrast micrograph); (d) *Thiodictyon elegans* (phase contrast micrograph); (e) *Thiopedia rosea* (phase contrast micrograph); and (f) *Thiocapsa pendens* (light-field micrograph, India ink preparation). Bar = 10  $\mu$ m. From Pfennig and Trüper (1992).

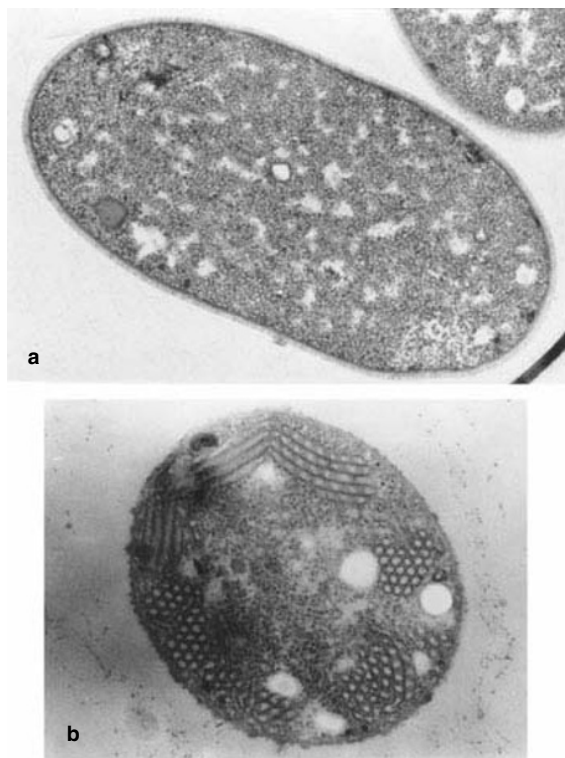


Fig. 3. Electron microscopic photomicrographs of ultrathin sections showing internal membrane structures of Chromatiaceae species. a) Cell of *Thiocystis gelatinosa* with vesicular membrane structures, and b) Cell of *Thiococcus pfennigii* with tubular internal membranes. (Courtesy of H. G. Trüper and J. B. Waterbury).

or  $\beta$  and carotenoids of the spirilloxanthin, the okenone or the rhodopinal groups. In some species, tetrahydrospirilloxanthin and derivatives thereof occur. In general, the color of cultures of strains with carotenoids of the spirilloxanthin group appear orange-brown to brownish-red or pink, of those with okenone purple-red, of those with tetrahydrospirilloxanthin peach-brown, and of those with carotenoids of the rhodopinal group purple-violet. The carotenoids absorb at 480–550 nm. Absorption bands of bacteriochlorophyll  $\alpha$  in vivo are at 380, 590–600, and 800–900 nm. Owing to the formation of different light-harvesting complexes, absorption spectra show a remarkable variation in the long wavelength range from 800–900 nm. Cultures of bacteria with bacteriochlorophyll  $\beta$  exhibit long wavelength absorption maxima at 980–1200 nm (Pfennig et al., 1997). Absorption spectra of whole cells are measured with cell suspensions washed twice in medium or appropriate salt solutions and then suspended in 60% sucrose solution (Biebl and Drews, 1969). Better results are often achieved by using isolated internal membranes suspended in buffer. For this purpose, it

is sufficient to break the cells by ultrasonication or with a French press and to separate whole cells and large cells fragments by centrifugation at 15,000  $\gamma$  from the internal membranes, which are used for spectral measurements. Although the color and the absorption spectra give first hints to the presence of certain carotenoids, it is emphasized that a careful chemical analysis is required for identification of these compounds.

In addition to the phenotypic characterization, information on the genetic relatedness of a new isolate has to be obtained. First, information on the phylogenetic relationship can be obtained by sequences of the 16S rDNA. For the description of a new species, the determination of the G+C content of the DNA is required. When the distinction of closely related strains and species has to be achieved, also DNA-DNA hybridization studies may be necessary. The DNA base ratio, expressed as the G+C content, has been found to span a large range (from 45.5–70.4 mol%) within the Chromatiaceae. Thus, species of the genus *Chromatium*, as known before the taxonomic rearrangements proposed by Imhoff et al. (1998) had values ranging from 48.0 to 70.4 mol% (Pfennig, 1989b). Because the G+C content is a crude measure of genomic relatedness of bacteria, these values already suggested enormous diversity within the genus *Chromatium*. A phylogenetic system would be expected to group bacteria together that have much less variation in their G+C content. Indeed, after rearrangement of the species of the Chromatiaceae according to their genetic relatedness on the basis of 16S rDNA sequences (Guyoneaud et al., 1998; Imhoff et al., 1998), a quite narrow range of the G+C content is found within most of the newly established genera. This is, e.g., approx. 63–66% in the cluster, including species of *Thiocapsa*, *Lamprocystis* and *Thiolamproyum*, 69–70% in *Marichromatium* species, 64–66% in *Halochromatium* species, and 48–50% in the true *Chromatium* species. This correlation appears of such significance, that any case of high intragenetic and intraspecies variation of the G+C content necessitates experimental examination. For example, the high intraspecies variation that is noted for *Allochromatium vinosum* (61.3–66.3), *Allochromatium warmingii* (55.1–60.2), and *Thiocystis violacea* (62.8–67.9) may suggest misclassification of some of the strains. Further studies are needed to clarify the situation.

Chemotaxonomic properties such as ring structure and the isoprenoid chain length of respiratory quinones and the cell membrane fatty acid composition (which has been found to be quite helpful for identification and classification of new isolates of purple nonsulfur bacteria; Hiraishi et al., 1984; Imhoff, 1984a; Imhoff and Bias-Imhoff, 1995) are of low diagnostic value



within the Chromatiaceae. Polar lipids have been analyzed so far in only a limited number of Chromatiaceae species. First results obtained with several freshwater species showed significant correlations with their new classification, i.e., almost identical polar lipid compositions were found 1) in *Allochromatium vinosum* and *Allochromatium warmingii*, 2) in all four *Thiocystis* species and 3) in the two *Chromatium* species (J. F. Imhoff, unpublished observation). Thus, the pattern of polar lipid composition may well turn out to be a relevant property to distinguish between genera of Chromatiaceae. Because of the phylogenetic basis of the taxonomy of the Chromatiaceae, this also implies that the polar lipid composition reflects phylogenetic relations. More analytical data on Chromatiaceae are required to support this concept.

For identification of known species, the properties listed in Table 1 may be used as a guide. For the detailed description of a new bacterium, more careful studies are required, including the utilization of substrates, relation to oxygen, the ability to grow in darkness, capability of respiratory and fermentative growth, vitamin requirement, as well as ranges and optima of salt concentration, pH and temperature.

## Preservation

For short- and medium-term preservation, stock cultures of purple sulfur bacteria are incubated at room temperature and a light intensity of 200–500 lux; they are grown until the initially formed elemental sulfur has just disappeared. According to Pfennig and Trüper (1992), freshly grown cultures have to be fed with 1 ml neutral sulfide solution to a final sulfide concentration of 1.5 mM and kept in the light for a few hours until the cells have formed intracellular globules of elemental sulfur. At this stage, the stock cultures can be stored in a refrigerator at 4–6°C for several months. The cultures keep well when they are put back into dim light at room temperature after 4–6 weeks of storage in the dark. Cultures with sulfur-free cells are then fed 1 ml of neutral sulfide solution and, after formation of elemental sulfur, put back into the refrigerator. Before transfer into fresh medium after 3–4 months of storage, the stock cultures are fed 1 ml of neutral sulfide solution and are transferred after the cells have formed elemental sulfur (milky appearance of the culture) and have started to grow.

For long-term storage, preservation in liquid nitrogen is recommended. Well grown cultures are supplemented with 50% dimethyl sulfoxide to give a final concentration of 5% (alternatively with a final content of 10% glycerol), thoroughly mixed, dispensed in 2-ml plastic ampules, sealed

and frozen in liquid nitrogen. Storage in liquid nitrogen is possible for many years.

## Physiology

Chromatiaceae are anoxygenic phototrophic bacteria that grow photolithoautotrophically under anoxic conditions in the light using sulfide or elemental sulfur as an electron donor. Many species are also able to use molecular hydrogen and thiosulfate as electron donors under reducing culture conditions, and some use reduced iron ions (Widdel et al., 1993; Ehrenreich and Widdel, 1994). Many species are strictly anaerobic and obligately phototrophic, others are capable of chemolithoautotrophic or chemorganoheterotrophic growth under microoxic to oxic conditions in the dark. Chromatiaceae are quite conservative in regard to the use of a limited number of simple organic carbon sources, of which acetate and pyruvate are the most widely used. Storage materials are polysaccharides, poly- $\beta$ -hydroxybutyrate, elemental sulfur, and polyphosphate. Vitamin B<sub>12</sub> is required by several species.

We distinguish two major physiological groups of Chromatiaceae, versatile and specialized species, respectively. The specialized species depend on strictly anoxic conditions and are obligately phototrophic. Sulfide is required, and thiosulfate and hydrogen are not used as electron donors. Only acetate and pyruvate (or propionate) are photoassimilated in the presence of sulfide and CO<sub>2</sub>. These bacteria do not grow with organic electron donors, chemotrophic growth is not possible, and sulfate is not assimilated as a sulfur source. Among these species are *Chromatium okenii*, *Chromatium weissei*, *Allochromatium warmingii*, *Isochromatium buderi*, *Thiospirillum jenense* and *Thiococcus pfennigii*.

The versatile species photoassimilate a larger variety of organic substrates. Most of them are able to grow in the absence of reduced sulfur sources with organic substrates as electron donors for photosynthesis and to assimilate sulfate as the sole sulfur source. Some species even grow chemoautotrophically or chemoheterotrophically (Gorlenko, 1974; Kondratieva et al., 1976; Kämpf and Pfennig, 1980; Kämpf and Pfennig, 1986). Among these species are *Allochromatium vinosum*, *Thiocystis violacea*, *Thiocapsa roseopersicina*, *Thiocapsa rosea*, *Thiocapsa pendens* and *Lamprobacter modestohalophilus*.

A comprehensive treatment of various aspects of the physiology of purple sulfur bacteria including structure, function and genetics of the photosynthetic apparatus is found in various chapters of *The Photosynthetic Bacteria* (Clayton and Sistrom, 1978) and *Anoxygenic Phototrophic*

*Bacteria* (Blankenship et al., 1995). A short overview on physiology and photosynthesis is given by Drews and Imhoff (1991). In the following, some basic principles and a few specific examples of metabolic properties of the Chromatiaceae are presented.

### Energy Generation and Photosynthesis

Anoxygenic photosynthesis depends on the presence of a complex membrane-bound photosynthetic apparatus, which includes reaction center and light harvesting (antenna) pigment-protein complexes. The reaction center and antenna proteins noncovalently bind bacteriochlorophylls, carotenoids and other cofactors in stoichiometric ratios. Most purple sulfur bacteria have two antenna complexes. The complexes of the reaction center are surrounded by core antenna (a B870 or B890 antenna complex with bacteriochlorophyll  $\alpha$ , and a B1020 complex with bacteriochlorophyll  $\beta$ ) and mostly by additional peripheral antenna (B800–850 complexes and in a few cases also B800–820 complexes with bacteriochlorophyll  $\alpha$ ).

All purple sulfur bacteria have an internal membrane system in which the photosynthetic apparatus including reaction center and antenna complexes is integrated. These internal membranes form vesicles or tubules and are interconnected to the cytoplasmic membrane. They can be isolated by cell rupture and fractionated centrifugation. Quite characteristically, the production of photosynthetic pigments, pigment-protein complexes, and photosynthetic membrane structures is suppressed by oxygen.

The principal function of the photosynthetic apparatus is the light-mediated excitation of a bacteriochlorophyll molecule in the reaction center, followed by charge separation, and resulting in electron transfer through the membrane. At first, light is harvested by antenna pigments (bacteriochlorophylls and carotenoids bound to proteins). When light energy is transferred from the antenna complex to the reaction center, one electron per light quantum is translocated from reaction center bacteriochlorophyll to bacteriopheophytin and further to an iron-ubiquinone complex, which bridges the gap between the inner membrane surface and the reaction center. The latter is located in the membrane, towards the outer surface. From the reduced iron-ubiquinone complex, the electron enters the intramembranous ubiquinone pool together with two protons (per quinone molecule and electron) from the cytoplasm. Ubiquinone acts as a mobile carrier within the membrane. The reduced ubiquinone travels to the  $bc_1$  complex, where the electron is donated to cytochrome  $c_2$ , and the two protons to the

topological outside of the cell. Cytochrome  $c_2$  acts as a mobile electron carrier between the  $bc_1$  complex and the reaction center complex outside of the cytoplasmic membrane. In the reaction center, it replaces the missing electron donated to the iron-ubiquinone complex. The proton gradient created this way between outside and inside drives ATP synthase, thus forming ATP in the cytoplasm (for details, see Drews, 1989; Drews and Imhoff, 1991).

For the generation of reduced nicotinamide adenine dinucleotide (NADH), Chromatiaceae and other phototrophic purple bacteria require external electron donors such as reduced sulfur compounds or hydrogen. The NADH formation requires reverse electron flow, which is driven by the proton motive force provided by the cyclic photosynthetic electron flow of the light reaction mechanism described above. The electrons required for  $NAD^+$  reduction are donated at less electronegative sites via  $c$ -type cytochromes. These electrons may derive from the oxidation of reduced sulfur compounds or molecular hydrogen.

As long as light is present, phototrophic purple bacteria are able to produce large amounts of ATP. In their natural environments, however, this is only the case during daytime. Therefore, the ability to make use of alternative mechanisms of energy conservation may be of selective advantage. Indeed, many purple sulfur bacteria not only are considerably tolerant to oxygen but also are able to perform respiratory energy transformations (Kondratieva et al., 1975; Kämpf and Pfennig, 1980).

### Hydrogen Metabolism

Hydrogen serves as an excellent photosynthetic electron donor for many purple sulfur bacteria and enables these bacteria to grow photolithoautotrophically. This capability was first detected in *Allochromatium vinosum* strain D (Gaffron, 1935; Roelofsen, 1935) and later in many other purple sulfur bacteria (see Sasikala et al., 1993; Vignais et al., 1995). Hydrogen uptake is catalyzed by a reversible, membrane-bound hydrogenase, which is induced by hydrogen and independent of the nitrogen source. This membrane-bound hydrogenase is not inhibited by ammonia, but strongly inhibited by CO. During dinitrogen-fixing growth conditions, this uptake hydrogenase recycles the hydrogen produced by nitrogenase. Mutants lacking this hydrogenase demonstrate an increased hydrogen production during dinitrogen fixation (see Drews and Imhoff, 1991). The “uptake” hydrogenases of phototrophic bacteria have been studied in much detail in *Allochromatium vinosum* and *Thiocapsa roseopersicina*, and the enzymes appear to

be membrane-bound and probably contain nickel and iron-sulfur clusters. In purple bacteria, the natural electron acceptors are cytochromes of the  $c$ - or  $\beta$ -type (Gogotov, 1978; Gogotov, 1984; Gogotov, 1986; Vignais et al., 1985).

A great number of phototrophic purple bacteria can photoproduce hydrogen under certain growth conditions. With dinitrogen, glutamate or aspartate as nitrogen source, a number of carbon substrates (lactate, acetate, butyrate, malate and others) may be completely transformed to  $\text{CO}_2$  and  $\text{H}_2$ , and these in turn may serve as substrates for photoautotrophic growth (Kondratieva and Gogotov, 1983; Sasikala et al., 1993; Vignais et al., 1995).

### Sulfur Metabolism

All Chromatiaceae species are capable of utilizing reduced sulfur compounds as photosynthetic electron donors. These are oxidized to sulfate as the final oxidation product. All of them oxidize sulfide and elemental sulfur, and some also oxidize thiosulfate and sulfite (Trüper, 1981a). During growth of Chromatiaceae on sulfide and thiosulfate, sulfur appears in the form of globules inside the bacterial cells. During oxidation of thiosulfate, the sulfur of these globules is entirely derived from the sulfane group of thiosulfate (Smith, 1965; Trüper and Pfennig, 1966). The sulfur in the globules exists in a metastable state and is not true elemental sulfur. It possibly consists of long chains of sulfur with polar ends either reduced (polysulfides) or oxidized (polythionates; Steudel, 1989; Steudel et al., 1990). The sulfur globules are surrounded by a protein monolayer consisting of three proteins in *Allochromatium vinosum* and two proteins in *Thiocapsa roseopersicina* (Brune, 1995a). Evidence is presented, that these sulfur globule proteins contain amino-terminal signal peptides pointing to an extracytoplasmic localization of the sulfur globules (Pattaragulwanit et al., 1998).

During aerobic dark growth, elemental sulfur may support respiration and serve as electron donor for chemolithotrophic growth (Breuker, 1964; Kämpf and Pfennig, 1986). During anaerobic dark, fermentative metabolism, intracellular sulfur serves as an electron sink during oxidation of stored carbohydrates and is reduced to sulfide (Van Gemerden, 1968b; Van Gemerden, 1968a; Van Gemerden, 1974). Though growth under these conditions is very poor in Chromatiaceae, several species have the capabilities of a fermentative metabolism that at least guarantees survival in the absence of light and oxygen (Van Gemerden, 1968a; Van Gemerden, 1968b; Krasilnikova et al., 1975; Krasilnikova, 1976; Krasilnikova et al., 1983).

### Enzymatic Reactions

Intermediates in enzymatic reactions of sulfide oxidation are sulfur and sulfite. While sulfur accumulates and is clearly visible in the cells, sulfite is not observed, possibly because of its cytoplasmic formation and immediate further oxidation to sulfate (Brune, 1995b). Thiosulfate oxidation is thought to proceed through the same intermediates as sulfide oxidation. Enzymes that catalyze oxidation of reduced sulfur compounds have been intensively studied and sulfur metabolism of phototrophic bacteria has been repeatedly discussed and reviewed (Trüper and Fischer, 1982; Trüper, 1984; Trüper, 1989; Brune, 1989; 1995b).

**FLAVOCYTOCHROME *c*** Present in several Chromatiaceae, flavocytochrome *c* can catalyze the electron transfer from sulfide to a variety of small *c*-type cytochromes, such as cytochrome *c*-550 of *Allochromatium vinosum* (Davidson et al., 1985). The subunits of the *Allochromatium vinosum* flavocytochrome have signal peptide leader sequences that are absent from the mature protein implying that the protein is located periplasmically (Dolata et al., 1993). Though flavocytochrome *c* may act in sulfide oxidation of some purple sulfur bacteria, it is absent in others, which strongly suggests that alternative routes of sulfide oxidation exist.

**SULFIDE QUINONE REDUCTASE** A role of sulfide quinone reductase, which has been characterized from cyanobacteria and purple nonsulfur bacteria (Brune, 1995b), in sulfide oxidation of Chromatiaceae has not been established so far.

**SULFITE REDUCTASE** Sulfite reductase present in *Allochromatium vinosum* is suggested to catalyze the oxidation of sulfide to sulfite. The enzyme contains siroheme as prosthetic group and is present in cells grown photoautotrophically but absent in those grown photoheterotrophically (Schedel et al., 1979). A role of this enzyme was proposed not only in the oxidation of sulfide but also of polysulfide and sulfur (Trüper, 1984). DNA-based evidence exists for the wider distribution of this gene among Chromatiaceae than anticipated so far from enzymatic activities tested in these organisms (Dahl et al., 1999).

**ADENOSINE-5'-PHOSPHOSULFATE REDUCTASE** Adenosine-5'-phosphosulfate (APS) reductase is involved in the oxidation of sulfite to sulfate. This enzyme, forming APS from sulfite and AMP, has been found in several Chromatiaceae (Trüper and Fischer, 1982). In most cases this enzyme is membrane-bound. Apparently it is not present in

all purple sulfur bacteria and was not found, e.g., in *Marichromatium gracile* and *Marichromatium purpuratum* (Trüper and Fischer, 1982). Because APS reductase is not present in all purple sulfur bacteria, alternative enzymatic reactions for the oxidation of sulfite have to be considered.

APS can react either with phosphate to yield adenosine diphosphate (ADP) and sulfate (ADP sulfurylase) or with pyrophosphate to yield adenosine triphosphate (ATP) and sulfate (ATP sulfurylase). These reactions allow energy conservation of the high-energy phosphate bond contained in APS. The enzyme ADP sulfurylase replaces the sulfate moiety of APS by inorganic phosphate, thus producing ADP, which can be disproportionated by adenylate kinase, leading to the formation of 1 ATP and 1 AMP per 2 ADP. These reactions have been found in several Chromatiaceae species (Trüper and Fischer, 1982).

**SULFITE OXIDOREDUCTASE** Sulfite oxidoreductase (sulfite: acceptor oxidoreductase) is an alternative enzyme, catalyzing the oxidation of sulfite to sulfate. It has been found in almost all purple sulfur bacteria examined (Trüper, 1981a; Trüper, 1989).

Sulfur from thiosulfate, which is readily oxidized by many Chromatiaceae, most likely enters the oxidative pathway at the level of sulfur and sulfite. Enzymatic activities of thiosulfate-sulfur transferases, which split thiosulfate to elemental sulfur and sulfite, have been measured in several Chromatiaceae (Trüper, 1984). In *Allochromatium vinosum* also, a thiosulfate: acceptor oxidoreductase, which forms tetrathionate from thiosulfate was found (Smith, 1966). Tetrathionate cannot be transformed further by *Allochromatium vinosum*. In the presence of sulfide, however, tetrathionate readily reacts chemically with the sulfide to form thiosulfate and sulfur (Podgorsek and Imhoff, 1999), both of which in turn can be oxidized by *Allochromatium vinosum*.

### Carbon Metabolism

In the Chromatiaceae, CO<sub>2</sub> is the most important carbon source. Under autotrophic growth conditions with CO<sub>2</sub> as sole carbon source, the Calvin cycle with ribulose biphosphate carboxylase (RubisCO) and phosphoribulokinase as key enzymes is employed (Tabita, 1995). Enzymological proof for the Calvin cycle exists for a number of species, *Allochromatium vinosum* (Fuller et al., 1961), *Chromatium okenii* (Trüper, 1964), *Thiocapsa roseopersicina* (Kondratieva et al., 1976; Zhukov, 1976), and *Thiococcus pfennigii* (Sahl and Trüper, 1977). There is little doubt, that all Chromatiaceae employ the Calvin cycle when

growing photolithoautotrophically (Kondratieva, 1979).

Already, van Niel (1931) demonstrated the stoichiometric linkage between photosynthetic carbon dioxide fixation and sulfide oxidation in phototrophic sulfur bacteria, and this relationship was experimentally confirmed for *Chromatium okenii* (Trüper, 1964). In the overall reaction, the reduction of two carbons from CO<sub>2</sub> to the oxidation level of carbohydrate is mediated by eight electrons derived from the oxidation of one sulfide to sulfate (Pfennig and Trüper, 1992).

The ability to use organic carbon sources is restricted to a low number of simple organic molecules in purple sulfur bacteria. A larger number of substrates is used in the more versatile species of the Chromatiaceae (e.g., *Allochromatium vinosum* and *Thiocapsa roseopersicina*) than in the specialized Chromatiaceae (e.g., *Chromatium okenii* and *Thiospirillum jenense*; Trüper, 1981b). The latter group is obligately photolithoautotrophic, but has a certain mixotrophic potential; these species strictly depend on the supply of CO<sub>2</sub> and sulfide, even if utilizing acetate or pyruvate. Acetate is assimilated by almost all purple sulfur bacteria.

### Nitrogen Metabolism

In principle, assimilatory nitrogen and sulfur metabolism is not different from that of related nonphototrophic bacteria. Ammonia, dinitrogen, and several organic nitrogen compounds (e.g., glutamate, aspartate or yeast extract) are the most appropriate nitrogen sources of most purple sulfur bacteria.

Ammonia is used as the preferred nitrogen source by all Chromatiaceae. As in many other bacteria, it is assimilated via glutamine synthetase and glutamate synthase reactions (Brown and Herbert, 1977). Nitrate is not utilized by Chromatiaceae. The majority of purple sulfur bacteria are able to fix dinitrogen, although in some species this capability is found only in certain strains (Madigan, 1995). As dinitrogen fixation is linked to hydrogen (gas) production, this field has received much attention in research (for reviews, see Vignais et al., 1985; Haselkorn, 1986; Hallenbeck, 1987; Ludden and Roberts, 1995; Madigan, 1995). Nitrogen metabolism has been much more intensively studied in purple nonsulfur bacteria compared to purple sulfur bacteria.

### Applications

Phototrophic purple sulfur bacteria have been and are being used in a number of biotechnolog-

ical processes. Most prominent examples of the application of phototrophic purple bacteria are their use in sewage treatment processes and for production of biopolymers and molecular hydrogen. Chromatiaceae have been used for the production of vitamin B<sub>12</sub> (Toohey, 1971; Koppenhagen, 1981a; Koppenhagen et al., 1981b) and biotin (Filippi and Vennes, 1971). As other phototrophic purple bacteria, they produce poly- $\beta$ -hydroxybutyrate and accumulate this in considerable amounts inside their cells (Liebergesell et al., 1991; Liebergesell et al., 1992). So far, no use has been made of the wide spectrum of colorful carotenoids produced by purple sulfur bacteria.

They have the potential to be used specifically for the removal of sulfide and for sulfur production from H<sub>2</sub>S-containing fluids and gases, though applications for such processes have not been reported.

The use of phototrophic sulfur bacteria in large-scale processes has the advantages that light is a clean energy source and oxygen is not required. However, the advantage of avoiding problems with oxygen supply, which is necessary for aerobic bacteria, is replaced by the disadvantage caused by the problems of supplying sufficient light. This is at least a problem in the lower latitudes and in regions with irregular periods of sunshine, because considerable efforts have to be made for artificial illumination. Under these circumstances, the high expenditure of energy and money considerably lowers the possible commercial acceptance of such processes.

### Waste Water Treatment

Phototrophic bacteria are regularly found in conventional sewage treatment plants, and though the facultative chemotrophic purple nonsulfur bacteria compete best under such conditions, purple sulfur bacteria always are present and under certain conditions form the dominant group (Holm and Vennes, 1971; Siefert et al., 1978).

Liquid wastes from food industries or communities often are fed into shallow open lagoons, which is a quite simple, but effective treatment system. Studies of such system have repeatedly shown the abundance of phototrophic sulfur bacteria: *Thiopedia rosea* in animal-fat-rendering waste, Chromatiaceae in petroleum refinery waste (Cooper, 1963; Cooper, 1965; Cooper et al., 1975), Chromatiaceae in municipal sewage (May and Stahl, 1967), *Thiocapsa roseopersicina* and *Allochrochromatium vinosum* in potato waste (Holm and Vennes, 1970), *Thiopedia rosea* in feedlot manure (Wenke and Vogt, 1981), and *Thiocapsa roseopersicina* in sugar factory efflu-

ents (N. Pfennig and H.G. Trüper, unpublished observations).

An advanced system using phototrophic bacteria in the purification of municipal and industrial wastewater is that developed by M. Kobayashi and coworkers working with mixed natural enrichments of phototrophic sulfur and nonsulfur bacteria (Kobayashi et al., 1971; Kobayashi and Tchan, 1973; Kobayashi, 1977; Kobayashi and Kobayashi, 1995). In addition to the organic solute and sulfide contents of a variety of sewages, phototrophic bacteria completely removed bad-smelling substances, such as putrescine, cadaverine, and mercaptans, as well as the carcinogen dimethylnitrosamine (Kobayashi and Tchan, 1978).

These authors also took advantage of the biomass produced by the phototrophic bacteria, which is a valuable source of animal feed owing to its rich content in vitamins and in essential and sulfur-containing amino acids (Vrati, 1984). It has been used in plankton production, in the culture of shrimp, and as food for fish and chicken (Kobayashi, 1977; Mitsui, 1979). Addition of phototrophic bacterial cells to the food increased the survival of fish as well as the production and quality of hens' eggs (Kobayashi and Tchan, 1973). With similar success, the cell biomass of phototrophic bacteria has been used as fertilizer in agriculture (Kobayashi and Tchan, 1973).

### Hydrogen Production

Under nitrogen starvation, almost all phototrophic bacteria are able to produce molecular hydrogen. This process is mainly due to hydrogen evolution from nitrogenase. A large number of substrates has been used by different research groups and with different purple bacteria to produce hydrogen (see Kumazawa and Mitsui, 1982; Cooper, 1963; Sasikala et al., 1993). Though most of this work has taken advantage of the purple nonsulfur bacteria, also purple sulfur bacteria were included in these studies (Mitsui, 1975, 1979). Bolliger et al. (1985) developed a system to produce hydrogen gas by phototrophic bacteria growing in sugar-refinery wastewater. Even purified and immobilized hydrogenase of *Thiocapsa roseopersicina* has been used for hydrogen production in biofuel cells (Yarapolov et al., 1982).

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## The Family Ectothiorhodospiraceae

JOHANNES F. IMHOFF

### Phylogeny

Separate phylogenetic lineages are represented by the purple sulfur bacteria belonging to the  $\gamma$ -Proteobacteria (Chromatiaceae and Ectothiorhodospiraceae) and by the purple nonsulfur bacteria, belonging to the  $\alpha$ -Proteobacteria and  $\beta$ -Proteobacteria.

Ectothiorhodospiraceae represent halophilic and alkaliphilic purple sulfur bacteria that form a separate lineage of phylogenetic descent related to the Chromatiaceae, as was first demonstrated by analysis of rRNA oligonucleotide catalogues (Stackebrandt et al., 1984). In a phylogenetic tree based on 16S rDNA sequences, both families form distinct groups within the  $\gamma$ -Proteobacteria (Imhoff and Süling, 1996).

The salt requirement not only is a characteristic property of all Ectothiorhodospiraceae, but also distinguishes moderately and extremely halophilic *Halorhodospira* species from slightly halophilic *Ectothiorhodospira* and *Thiorhodospira* species. Two clearly distinct phylogenetic lineages within the Ectothiorhodospiraceae separate the slightly halophilic and marine species from the moderately to extremely halophilic species (see Fig. 1).

Correlation between salt relations and phylogenetic position of the purple sulfur bacteria point out the phylogenetic and taxonomic importance of salt requirement and salt dependence, which also distinguishes major phylogenetic groups of the Chromatiaceae (Imhoff et al., 1998b) and genera of the purple nonsulfur bacteria such as *Rhodobacter* and *Rhodovulum* (Hiraishi and Ueda, 1994) and others (Imhoff et al., 1998a). At present, *Arhodomonas aquaeolei* is the only nonphototrophic, purely chemotrophic representative in this lineage, which is closely related to *Halorhodospira* species (Adkins et al., 1993; Imhoff and Süling, 1996).

### Taxonomy

#### *Ectothiorhodospiraceae*

The taxonomic position of the Ectothiorhodospiraceae has been disputed since their discovery by Pelsh, 1936. He distinguished phototrophic purple sulfur bacteria that deposit elemental sulfur globules outside the cells, which he called “Ectothiorhodaceae,” from those with elemental sulfur inside their cells, which he called “Endothiorhodaceae” (Pelsh, 1937). Pelsh’s isolates were lost, but *Ectothiorhodospira mobilis* was reisolated (Trüper, 1968), and the genus *Ectothiorhodospira* included into the Chromatiaceae (Pfennig and Trüper, 1971) because of the ability to perform an oxidative dissimilatory sulfur metabolism similar to other phototrophic purple sulfur bacteria. Later, a clear differentiation of *Ectothiorhodospira* species from Chromatiaceae was demonstrated by oligonucleotide patterns of 16S rRNA molecules (Stackebrandt et al., 1984), substantiated by 16S rDNA sequence analyses (Imhoff and Süling, 1996), and a separation of the Ectothiorhodospiraceae as a family distinct from the Chromatiaceae was proposed (Imhoff, 1984a). The complete sequence analysis of the 16S rDNA from a large number of strains including all available type strains of *Ectothiorhodospira* and *Halorhodospira* not only supported their separation from the Chromatiaceae but also demonstrated the divergence of two groups within this family (Imhoff and Süling, 1996). Because of the large phylogenetic distance between these two groups, the extremely halophilic species were transferred to the new genus *Halorhodospira* (Imhoff and Süling, 1996). The separation of the two genera of Ectothiorhodospiraceae is supported by a number of physiological properties and chemotaxonomic characteristics. Studies on DNA-DNA and rRNA-DNA hybridization (Ivanova

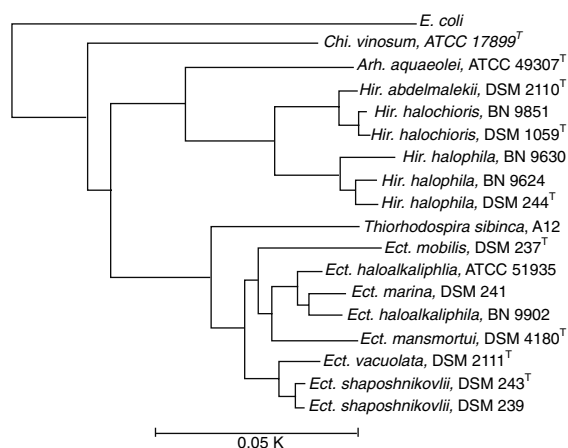
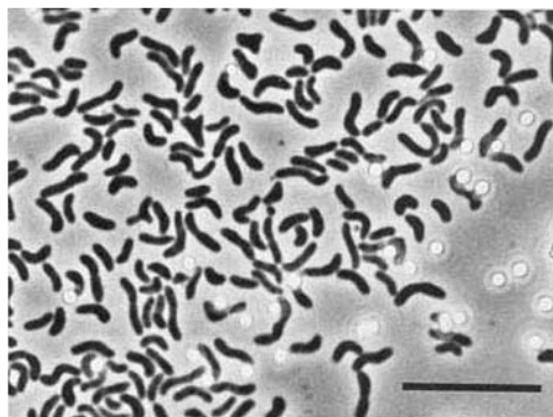


Fig. 1. Phylogenetic relatedness of the Ectothiorhodospiraceae derived from 16S rDNA sequences (Imhoff and Süling, 1996; Bryantseva et al., 1999).

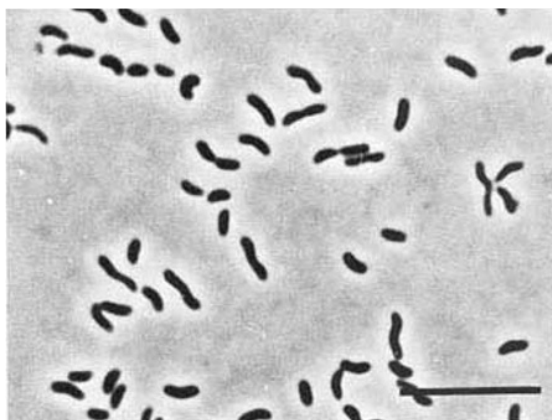
et al., 1985), analysis of lipopolysaccharides (Zahr et al., 1992), DNA restriction patterns (Ventura et al., 1993), as well as lipid, fatty acid and quinone composition (Imhoff et al., 1982; Imhoff 1984b; Thiemann and Imhoff, 1996; Tindall, 1980; Ventura et al., 1993; see Imhoff and Bias-Imhoff, 1995) support the phylogenetic and taxonomic relations within the Ectothiorhodospiraceae. The taxonomy of Ectothiorhodospiraceae is in accord with their phylogenetic relationships based on 16S rDNA sequences.

*Ectothiorhodospira* and *Halorhodospira* species are rods, most often slightly bent rods, vibrios or spirilla that during oxidation of sulfide produce elemental sulfur which is deposited outside the cells (Fig. 3, Tables 2 and 3). Only one species, *Ectothiorhodospira vacuolata*, is known to produce gas vesicles (Fig. 4). All of the phototrophic Ectothiorhodospiraceae produce characteristic internal membrane stacks that may constitute a large fraction of the internal volume of these cells (Figs. 5 and 6).

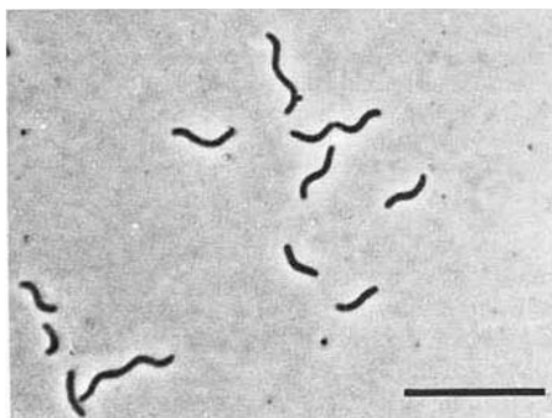
In recent years two new bacteria have been described which are phylogenetically related to the Ectothiorhodospiraceae. *Arhodomonas aquaeolei* is a moderately halophilic, strictly chemotrophic bacterium that is distantly related to *Halorhodospira* species, but clearly clusters with the branch of moderately and extremely halophilic bacteria of this family (Adkins et al., 1993; Imhoff and Süling, 1996). *Thiorhodospira sibirica* is a strictly phototrophic bacterium phylogenetically related to *Ectothiorhodospira* species and belonging to the branch of slightly halophilic Ectothiorhodospiraceae (Bryantseva et al., 1999; Fig. 1). It is unique in depositing elemental sulfur not only outside the cells but also in the peripheral part of the cells. Diagnostic properties of the genera *Ectothiorhodospira*,



a



b



c

Fig. 2. Cell morphology of (a) *Ect. mobilis* DSM 237; (b) *Hlr. halophila* BN 9621; (c) *Hlr. halochloris* ATCC 35916. Note the large and small sulfur globules in (a) and (c). Bar = 10  $\mu$ m.

*Halorhodospira* and *Thiorhodospira* are set forth in Table 1.

### *Ectothiorhodospira*

This genus is represented by slightly halophilic and marine species some of which may tolerate



Fig. 3. Phase contrast photomicrograph showing cell morphology of *Ect. vacuolata* DSM 2111 containing large and refractile gas vesicles in the cells.

moderately high salt concentrations. Growth is dependent on saline and alkaline conditions. All species of this genus are rods or spirals, motile by a polar tuft of flagella, have internal photosynthetic membranes as lamellar stacks (Cherni et al., 1969; Holt et al., 1968; Imhoff et al., 1981; Imhoff and Süling, 1996; Remsen et al., 1968; Oren et al., 1989), and contain menaquinone with 7 isoprene units (MK-7) together with ubiquinones Q-7 or Q-8 (Imhoff and Bias-Imhoff, 1995).

Analyses of 16S rDNA sequences and fatty acid composition supported the identification and species assignment of some strains, but demonstrated misclassification of others (Thiemann and Imhoff, 1996; Imhoff and Süling, 1996). The close relationship between *Ect. shaposhnikovii* and *Ect. vacuolata* that was demonstrated already by DNA-DNA hybridization studies (Ivanova et al., 1985) was supported. Several strains which previously had been tentatively

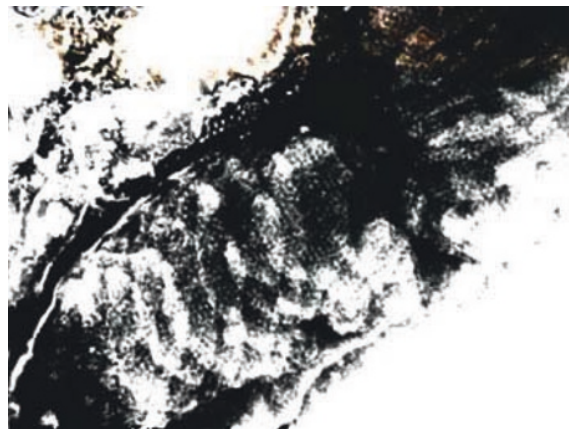


Fig. 5. Electron photomicrograph of a negatively stained cell of *Hlr. halochloris* showing regular structures in the internal stacks of photosynthetic membranes.



Fig. 4. Electron photomicrograph of a negatively stained cell of *Hlr. halophila* showing the extension of large internal stacks of photosynthetic membranes.

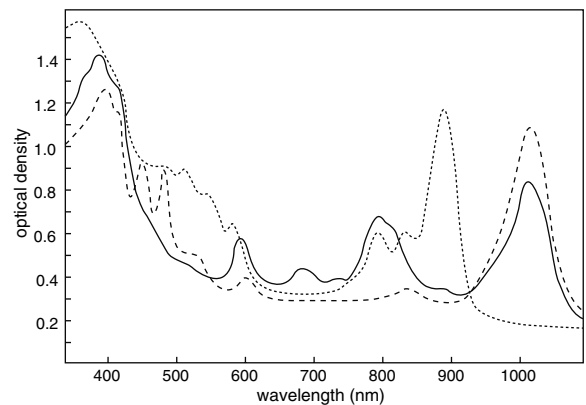


Fig. 6. Absorption spectra of chromatophore suspensions of *Halorhodospira halochloris* (—), *Halorhodospira halophila* (.....) and *Blastochloris sulfoviridis* (-----).

Table 1. Differential characteristics of the phototrophic Ectothiorhodospiraceae.

	<i>Halorhodospira</i>	<i>Ectothiorhodospira</i>	<i>Thiorhodospira</i> <sup>d</sup>
Gram-stain	negative	negative	negative
Phylogenetic group	gamma-Proteobacteria	gamma-Proteobacteria	gamma-Proteobacteria
Flagellation	bipolar flagella	polar tuft of flagella	polar tuft
Internal membranes	lamellar stacks	lamellar stacks	lamellar stacks
Cell form	vibrioid to spirilloid	vibrioid to spirilloid	vibrioid to spirilloid
Cell width (µm)	0.5–1.2	0.7–1.5	3–4
Growth medium <sup>a</sup>	EM 15–30%	EM 5% or mMPM	mMPM
Optimum salinity	15–25%	1–7%	0.5–1.0%
Important osmotica	glycine betaine, ectoine, trehalose	glycine betaine, sucrose	n.d.
Fatty acid cluster <sup>b</sup>	I–IV	V–VII	n.d.
Major quinone components <sup>c</sup>	MK8, Q8 and MK4/5	MK7 and Q7 or Q8	n.d.
Mol% G + C of DNA	50.5–69.7	61.4–68.4	56.0–57.4

<sup>a</sup>EM: standard medium for haloalkaliphilic Ectothiorhodospiraceae species according to Imhoff (1988a) with varying salt concentrations; mMPM: modified marine Pfennig's medium according to Trüper (1970);

<sup>b</sup>Fatty acid clusters according to Thiemann and Imhoff (1995);

<sup>c</sup>Major quinone components according to Imhoff (1984b) and Ventura et al. (1993);

<sup>d</sup>Data on *Thiorhodospira* are from Bryantseva et al. (1999).

identified as *Ectothiorhodospira mobilis* formed a separate cluster on the basis of their 16S rDNA sequences and were recognized as two new species. *Ectothiorhodospira haloalkaliphila* includes the most alkaliphilic strains originating from strongly alkaline soda lakes, strain 51/7 (BN 9903, ATCC 51935) and C (BN 9902, Imhoff et al., 1978). In particular, strain BN 9903 (ATCC 51935) has been intensively studied (Imhoff et al., 1982; Imhoff, 1984b; Imhoff and Riedel, 1989; Imhoff and Thiemann, 1991; Imhoff et al., 1991; Stackebrandt et al., 1984; Zahr et al., 1992; Ventura et al., 1993) and it is now quite clear that this strain does not belong to *Ect. mobilis* (Imhoff and Süling, 1996). *Ectothiorhodospira marina* is represented by isolates from the marine environment (strain BA 1010, DSM 241; Matheron and Baulaigue, 1972). Another isolate, strain BA 1011 (Matheron and Baulaigue, 1972) was most similar to strain BA 1010, but due to the lack of sufficient evidence its identification on the species level to strain BA 1010 was kept open (Imhoff and Süling, 1996).

Quite a number of strains were confirmed as strains of *Ect. mobilis*, namely strains 8112 (DSM 237), 8113 (DSM 238), and 8815 (DSM 240, Trüper, 1970; Mandel et al., 1971). Others that were tentatively assigned to this species were not identical with *Ect. mobilis*. Strain 8115 (DSM 239, Trüper, 1970; Mandel et al., 1971) was recognized as belonging to *Ect. shaposhnikovii*, as supported by DNA-DNA hybridization studies (Ivanova et al., 1985).

Presently known species of this genus are *Ect. mobilis*, *Ect. marismortui*, *Ect. marina*, *Ect. haloalkaliphila*, *Ect. shaposhnikovii* and *Ect. vacuolata*. Diagnostic properties of these species are shown in Table 2.

### *Halorhodospira*

This genus represents moderately and extremely halophilic species that were removed from the genus *Ectothiorhodospira* (Imhoff and Süling, 1996). All *Halorhodospira* species are vibrioid or spirals, motile by bipolar flagella and have internal photosynthetic membranes as lamellar stacks (Imhoff and Süling, 1996; Imhoff and Trüper, 1977, 1981; Raymond and Sistro, 1967, 1969). Growth is dependent on highly saline and alkaline conditions.

In agar media, red- or green-colored colonies are formed. Photosynthetic pigments of the red-colored species are bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series, with spirilloxanthin as the predominant component (Schmidt and Trüper, 1971; Schmidt, 1978). The green-colored species, *Hlr. halochloris* and *Hlr. abdelmalekii*, contain bacteriochlorophyll *b* esterified with  $\Delta$ -2,10-phytadienol, not with phytol as in the bacteriochlorophyll *a* of *Hlr. halophila* (Steiner et al., 1981; Steiner, R., personal communication). The carotenoid content of *Hlr. halochloris* and *Hlr. abdelmalekii* is low. The carotenoid composition of both of these species is quite similar. Mainly methoxyrhodopin glucoside (major), rhodopin glucoside, and rhodopin have been found in *Hlr. halochloris* (Schmidt, K., personal communication). Absorption spectra of living cells or chromatophore suspensions allow the identification of the bacteriochlorophyll present and provide some information on the types of carotenoids (Fig. 2).

Presently known species of this genus are *Halorhodospira halophila*, *Halorhodospira halochloris* and *Halorhodospira abdelmalekii*.

Table 2. Differential characteristics of *Ectothiorhodospira* species.

Differential characteristics of <i>Ectothiorhodospira</i> species						
	<i>Ect. mobilis</i>	<i>Ect. marismortui</i>	<i>Ect. marina</i>	<i>Ect. haloalkaliphila</i>	<i>Ect. shaposhnikovii</i>	<i>Ect. vacuolata</i>
Cell diameter (µm)	0.7–1.0	0.9–1.3	0.8–1.2	0.7–1.2	0.8–0.9	1.5
Motility	+	+	+	+	+	+
Color of cell suspensions	red	red	red	red	red	red
NaCl optimum (%)	2–3%	3–8%	2–6%	5%	3%	1–6%
Salinity range (%)	1–5%	1–20%	0.5–10.0	2.5–15%	0–7%	0.5–10.0%
pH optimum	7.6–8.0	7.0–8.0	7.5–8.5	8.5–10.0	8.0–8.5	7.5–9.5
Growth medium	mPF	mPF	mPF	mPF/EM5%	mPF/EM5%	mPF/EM5%
Chemolithotrophic growth	–	–	0	+	+	0
Sulfate assimilation	+	–	(+)	+	+	–
FA cluster	V	V	VII	VII	VI	VI
Major quinones	MK7/Q8	MK7/Q8	MK7/Q8	MK7/Q8	MK7/Q7	MK7/Q7
Mol% G + C of DNA (type strain)	67.3–68.4 67.3 (Bd)	65.0 65.0 (T <sub>m</sub> )	62.8 62.8 (Bd)	62.2–63.5 63.5 (T <sub>m</sub> )	62.0–64.0 62.0 (T <sub>m</sub> )	61.4–63.6 63.6 (T <sub>m</sub> )

Substrates used						
Hydrogen	+	–	0	0	+	+
Sulfide	+	+	+	+	+	+
Thiosulfate	+	–	+	+	+	+
Sulfur	+	+	+	+	+	+
Sulfite	+	0	+	0	+	0
Acetate	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+
Propionate	+/-	(+)	+	0	+	+
Butyrate	+/-	–	–	0	+	–
Lactate	+/-	(+)	+	0	+	–
Fumarate	+	+	+	+	+	+
Succinate	+	+	+	+	+	+
Malate	+	+	+	+	+	+
Fructose	+/-	–	–	–	+	(+)
Glucose	+/-	–	–	–	0	–
Ethanol	–	–	–	–	–	–
Propanol	–	–	–	–	–	–

none of the species uses formate, methanol, ethanol, propanol, citrate, benzoate.

Diagnostic properties of *Halorhodospira* species are shown in Table 3.

### *Thiorhodospira*

A new phototrophic sulfur bacterium isolated from a Siberian soda lake, *Thiorhodospira sibirica*, has been shown to be genetically related to the genus *Ectothiorhodospira* and many of its properties resemble those of *Ectothiorhodospira* species (Bryantseva et al., 1999). During photo-lithoautotrophic growth with sulfide as electron donor, the impressively large bacterium forms elemental sulfur globules outside as well as inside the cells. Upon careful microscopic inspection, it can be realized that the sulfur globules associated with the cells are located in the periphery that is represented by an apparently large periplasmic space, but not in the cell interior. Therefore, the mechanism of elemental sulfur formation and the location of the involved

enzymes appear to be the same as in species of other *Ectothiorhodospiraceae*.

In the presence of sulfide, organic substances are photoassimilated. Cells are vibrioid or spiral and motile by a monopolar flagellar tuft. Internal photosynthetic membranes are parallel lamellae underlying the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series. Development is dependent on sodium salts in low concentrations and on alkaline conditions. The metabolism is strictly anaerobic and obligately phototrophic.

### *Arhodomonas*

*Arhodomonas aquaeolei* has been found to be genetically related to the *Ectothiorhodospiraceae* and in particular to *Halorhodospira* species (Adkins et al., 1993; Imhoff and Süling, 1996). It is the first halophilic and strictly



Table 3. Differential characteristics of *Halorhodospira* species.

Differential characteristics of <i>Halorhodospira</i> species			
	<i>Hlr. halophila</i>	<i>Hlr. abdelmalekii</i>	<i>Hlr. halochloris</i>
Cell diameter (µm)	0.6–0.9	0.9–1.2	0.5–0.6
Motility	+	+	+
Color of cell suspensions	red	green	green
Bchl	a	b	b
NaCl optimum (%)	11–32%	12–18%	14–27%
pH optimum (%)	8.5–9.0	8.0–9.2	8.1–9.1
Sulfate assimilation	–	–	+
FA cluster	I + II	III	IV
Major quinones	MK8/Q8*	MK8/Q8*	MK8/Q8*
Mol% G + C of DNA (type strain)	66.5–69.7 68.4 (Bd)	63.3–63.8 63.8 (T <sub>m</sub> )	50.5–52.9 52.9 (T <sub>m</sub> )
Substrates used			
Hydrogen	0	0	0
Sulfide	+	+	+
Thiosulfate	+	–	–
Acetate	+	+	+
Pyruvate	+	+	+
Propionate	+	+	+
Lactate	+/-	–	–
Fumarate	+	+	+
Succinate	+	+	+
Malate	+	+	+

no species uses formate, butyrate, glucose, fructose, glycerol, methanol, ethanol, propanol.

\*In addition a short chain isoprenoid quinone is present in large amounts.

chemotrophic bacterium that is a close relative to the otherwise phototrophic Ectothiorhodospiraceae and has a restricted spectrum of carbon sources used for growth. Primarily due to its genetic relationship based on the 16S rDNA sequence, it is considered as a member of the Ectothiorhodospiraceae.

## Habitats

Like other phototrophic sulfur bacteria, phototrophic Ectothiorhodospiraceae (the strictly chemotrophic *Arhodomonas* is not considered here) develop under anoxic conditions, if soluble sulfide, light and bicarbonate are available. Accordingly they inhabit anoxic environments with sulfide and light. Phototrophic Ectothiorhodospiraceae are found in alkaline environments with saline or hypersaline conditions. The salt requirement of species belonging to the phylogenetic lineage of *Ectothiorhodospira* and *Thiorhodospira* is low and characteristic of bacteria from brackish and marine environments. A requirement for moderate to extreme salt concentrations specifies bacteria of the lineage with *Halorhodospira* [and *Arhodomonas*] species. Their requirement for and the tolerance of salt and alkaline pH is the physiological prerequisite to inhabit soda lakes and alkaline brines,

which are the natural habitats to which these bacteria are specifically adapted. Apparently, ecological aspects and adaptation of bacteria to specific factors of their natural environment are of importance in phylogeny and taxonomy (Fig. 1). Distinct phylogenetic lineages are expected to result from the evolution in separate habitats that, due to their outstanding properties, allow the development of specifically adapted bacteria only. Such outstanding properties are e.g., extreme conditions of salt concentration, temperature and pH.

Phototrophic species of the Ectothiorhodospiraceae were isolated from marine sources, estuaries, salt flats, salt lakes, and soda lakes from many parts of the world (Grant et al., 1979; Imhoff and Trüper, 1976, 1977, 1981; Imhoff et al., 1978, 1979, 1981; Matheron and Baulaigue, 1972; Pelsh, 1936; Raymond and Sistro, 1967; Tew, 1966; Tindall, 1980; Trüper, 1968, 1970; Ventura et al., 1988; see Imhoff, 1988b). Occasionally, certain *Ectothiorhodospira* species may be found in soil.

Highly saline and alkaline soda lakes show a natural abundance of *Halorhodospira* species (Jannasch, 1957; Imhoff et al., 1978, 1979; Imhoff, 1988b), which can be taken as proof of their successful adaptation to these environments. Because of their mass development, these bacteria may cause red or green coloration of their



Fig. 7. Shallow shore line of a soda lake in the Wadi Natrun (Egypt) with massive development of *Hlr. halophila* causing intense red coloration.

habitats. Shallow parts of soda lakes in the Wadi Natrun may be intensively red colored, due to the development of *Hlr. halophila* (Fig. 7). Small puddles of such locations may show separate development of green- and red-colored species of *Halorhodospira* (Fig. 8). Also top layers of the sediments of Wadi Natrun lakes demonstrated separate layers of the green-colored and the red-colored *Halorhodospira* species (Fig. 9). *Hlr. abdelmalekii*, *Hlr. halochloris*, and in particular *Hlr. halophila* are among the most halophilic phototrophic bacteria (see Imhoff, 1988b). Isolates of *Hlr. halophila* from soda lakes in the Wadi Natrun (Egypt), which have salt optima of 25% total salts and can grow in saturated salt solutions, are the most halophilic and halotolerant eubacteria.

*Ectothiorhodospira marismortui* was isolated from a hypersaline sulfur spring on the shore of the Dead Sea (Oren et al., 1989). The spring water was characterized by high salinity (approx. 17%), high sulfide content (approx. 2.5 mM), nearly 40°C and a pH around 5.2. The isolate grew poorly at pH 5.5 but showed optimal development between pH 7 and 8 (Oren et al., 1989). *Thiorhodospira sibirica* was found in microbial

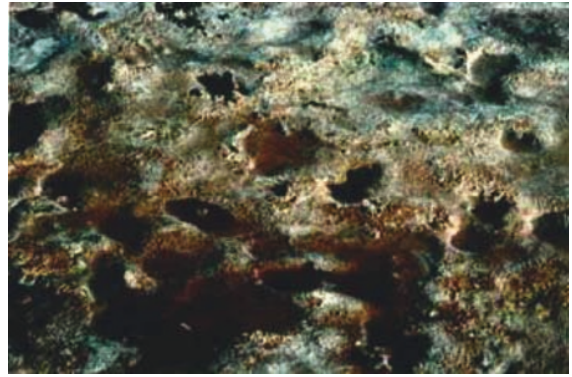


Fig. 8. Soda Lake Muluk in the Wadi Natrun (Egypt), which was almost dry due to falling water level in the area. Distinct red- and green-colored puddles can be seen, in which red- and green-colored species of the genus *Halorhodospira* predominate, and have been isolated (Imhoff et al., 1979).

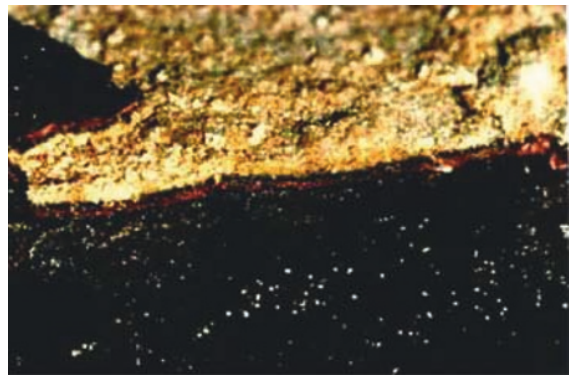


Fig. 9. Separate development of green-colored and red-colored *Halorhodospira* species within the top layers of a sediment of an alkaline soda lake of the Wadi Natrun (Egypt), the green species being on top.

films formed on the sediment surface or on decaying plant material in the littoral of Siberian soda lakes with low salinity (0.2–1%) and alkaline pH (pH 9.2–9.7; Bryantseva et al., 1999).

## Isolation

### Selective Enrichment

Marine strains of *Ectothiorhodospira* have been selectively enriched under photoautotrophic conditions in modified Pfennig's medium (Pfennig, 1965; Trüper, 1970; Pfennig and Trüper, 1992) with sulfide as an electron donor, and under saline and alkaline conditions (3% NaCl and pH 7.6 to 8.5), even in the presence of high numbers of marine Chromatiaceae in the natural sample (Trüper and Imhoff, 1981).

For the extremely halophilic *Halorhodospira* species, the dependence on and the tolerance of

high salinity and alkalinity are strongly selective conditions for their enrichment. Many *Halorhodospira* strains have been isolated from various locations using a medium [HSE (Highly Saline Ectothiorhodospiraceae) medium] based on the mineral salt composition of the soda lakes of the Wadi Natrun (Jannasch, 1957; Imhoff and Trüper, 1977; Imhoff et al., 1979; Imhoff, 1988a) and modifications thereof. Depending on the species composition of the sample and the salt concentration of the media, various Ectothiorhodospiraceae will develop.

For enrichment, environmental samples are inoculated into suitable media in 20-ml screw-cap test tubes or 50-ml bottles, which are completely filled with the medium. They are incubated at the desired temperature and under illumination of an incandescent lamp at 1,000 to 10,000 lux.

### Isolation Procedures

**Preparation of Agar Dilution Series.** For isolation, agar dilution series are prepared either with enrichment cultures or with promising natural samples by direct inoculation from the environment. Media used for direct isolation without prior enrichment does not need to be selective.

In a modification of the method of Pfennig (Pfennig 1965; Trüper, 1970), purified agar is dissolved (1.8%) in distilled water and distributed in amounts of 3 ml into cotton-plugged test tubes. The agar is sterilized by autoclaving. The liquid agar is kept at 50°C in a water bath until used. A suitable medium is placed in the same water bath, and 6 ml of the pre-warmed medium is added to each test tube. Medium and agar are mixed thoroughly by turning the tubes upside down and back, and kept at 50°C. Six to eight tubes are sufficient for each dilution series. The first tube is inoculated with a natural sample or enrichment culture and mixed carefully; approximately 0.5 to 1.0 ml is transferred to a second tube, mixed carefully, and the procedure continued up to the last tube. The tubes are immediately placed into a cold water bath until the agar has hardened, then they are sealed with a paraffin mixture (3 parts paraffin oil and 1 part paraffin) and kept in the dark for several hours before illumination with approx. 500 to 2000 lux. After cells have grown to visible colonies, the paraffin layer is removed by melting, and well-separated colonies are picked with a Pasteur pipette (the tip drawn out to a thin capillary) and transferred to a second dilution series. In general, three to four such dilution series are necessary to obtain pure cultures. When pure cultures have been obtained, single colonies are inoculated into liquid medium.

**Isolation on Agar Plates.** A convenient method for growing phototrophic sulfur bacteria on agar plates has been described by Irgens, 1983. Media without added sulfide are used with the GasPak system (Becton Dickinson and Co., Cockeysville, MD). As a source of sulfide, a test tube with 0.05 to 0.1 g of thioacetamide and 1 ml of 0.5 N HCl is placed in the anaerobic jar. Thioacetamide slowly decomposes in acid to ammonia, hydrogen sulfide, and acetic acid. The H<sub>2</sub>S gas is released over a period of at least one week. As indicators, methylene blue (for oxygen) and a strip with lead acetate (for H<sub>2</sub>S) are included. This method also can be used conveniently to assay viable counts of purple sulfur bacteria. It has been used successfully for the isolation of species of the genera *Pfennigia*, *Allochroamtium*, *Lamprocystis*, *Thiocapsa*, *Thiocystis*, and *Ectothiorhodospira* (Irgens, 1983).

### Media for *Ectothiorhodospira* Species

Enrichment and subsequent isolation of *Ect. mobilis*, *Ect. shaposhnikovii* and *Ect. marina* have been achieved by using Pfennig's medium for purple sulfur bacteria with 3% NaCl at alkaline pH 7.6–8.5 (Pfennig, 1965; Trüper, 1970; Matheron and Baulaigue, 1972). This medium apparently supports growth of all *Ectothiorhodospira* species including *Ect. marismortui* (Thiemann and Imhoff, 1996). It is not suitable for the extremely halophilic species *Hlr. halochloris*, *Hlr. halophila*, and *Hlr. abdelmalekii*. A strongly alkaline medium that is used for *Halorhodospira* species (HSE medium), at salt concentrations of 3–5% also supports growth of several *Ectothiorhodospira* species, namely *Ect. haloalkaliphila*, *Ect. vacuolata* and *Ect. shaposhnikovii*, but not of *Ect. mobilis*, *Ect. marismortui* and *Ect. marina* (Thiemann and Imhoff, 1996; Table 2).

### HSE (Highly Saline Ectothiorhodospiraceae) Medium for *Halorhodospira* Species

An alkaline and highly saline medium based on the mineral salts composition of the soda lakes of the Wadi Natrun has been used for isolation of *Ectothiorhodospira* and *Halorhodospira* strains from various environments (Imhoff and Trüper, 1977, 1981; Imhoff et al., 1978, 1981). This medium is well suited for isolation and cultivation of all *Halorhodospira* species and several *Ectothiorhodospira* species. It is buffered by bicarbonate (200 mM, pH 9.0) and adjusted to the desired salinity by changing the concentration of NaCl. Also the type strain of *Hlr. halophila*, SL1, (Raymond and Sistro, 1967) grows well in this medium. The mineral salts composi-

tion of this medium (Imhoff and Trüper, 1977) and its preparation as recommended for standard cultivation procedures is as given below (see Imhoff, 1988a).

The basal medium has the following composition (for a medium with 15% salinity, per liter):

KH <sub>2</sub> PO <sub>4</sub>	0.8 g
Na acetate	2.0 g
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> · 15H <sub>2</sub> O	1.0 g
NaCl	130.0 g
1 M Na carbonate, pH 9.0	200.0 ml
Trace element solution SLA (see below)	1 ml

The components are dissolved in 600 ml of distilled water and the carbonate buffer is added. The volume is brought to 980 ml and the solution is autoclaved. The salinity is adjusted to the desired value by variation of the NaCl content (assuming a contribution of 2% by the other medium constituents). The final pH is 9.0.

The following salt solutions are autoclaved separately and, after cooling, they are added with gentle stirring.

2% MgCl <sub>2</sub> · 7H <sub>2</sub> O	5 ml/liter
1% CaCl <sub>2</sub> · 2H <sub>2</sub> O	5 ml/liter
5% Na <sub>2</sub> S · 9H <sub>2</sub> O	5–10 ml/liter
20% NH <sub>4</sub> Cl	4 ml/liter

The medium is immediately dispensed into sterilized culture vessels, which are filled with medium except for a small air bubble. The vessels are incubated at the desired incubation temperature (33–400°C) prior to inoculation to achieve volume expansion and then 10% of the medium is replaced by a fresh preculture.

For *Halorhodospira halophila* and most *Ectothiorhodospira* species, thiosulfate is a suitable additional electron donor that makes feeding with sulfide unnecessary. Thiosulfate can be omitted in media for species that do not use thiosulfate, such as the green-colored *Halorhodospira* species. Acetate is a suitable carbon source for all species and increases the cell yield of the cultures.

Trace element solution SLA (Imhoff and Trüper, 1977)

FeCl <sub>2</sub> · 4H <sub>2</sub> O	1.800 mg
CoCl <sub>2</sub> · 6H <sub>2</sub> O	250 mg
NiCl <sub>2</sub> · 6H <sub>2</sub> O	10 mg
CuCl <sub>2</sub> · 2H <sub>2</sub> O	10 mg
MnCl <sub>2</sub> · 4H <sub>2</sub> O	70 mg
ZnCl <sub>2</sub>	100 mg
H <sub>3</sub> BO <sub>3</sub>	500 mg
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	30 mg
Na <sub>2</sub> SeO <sub>3</sub> · 5H <sub>2</sub> O	10 mg

The salts are dissolved separately in a total of 900 ml of double-distilled water; the solutions are mixed, the pH is adjusted to 2–3 with 1 N HCl, and the final volume is brought to 1 liter.

## Cultivation

In general, cultivation of *Ectothiorhodospira* species is possible in any size of bottle and glass

fermenter as long as illumination is sufficient and temperature is controlled. For routine cultivation 50-ml or 100-ml bottles appear the most suitable size. Because of the problems of self-shading of the cells, even for growth of larger cell masses round 1-liter bottles are very well suited. Cultures are incubated at 1,000 to 20,000 lux (approximately 100 to 2,000 foot candles) and at temperatures between 33 and 40°C. The pH optima found with different strains and species (and also in different media) are between 7.4 and 9.1. Salt optima of the different species are shown in Tables 2 and 3. For routine cultivation, the HSE medium is used at salinities of 15% for *Hlr. abdelmalekii*, *Hlr. halochloris*, and some strains of *Hlr. halophila* (including the type strain), of 25% for the most halophilic strains of *Hlr. halophila*, of 5% for *Ect. haloalkaliphila* and of 2–3% for *Ect. shaposhnikovii* and *Ect. vacuolata*. Other *Ectothiorhodospira* species are routinely grown with modified Pfennig's medium containing 3% NaCl.

## Identification

*Ectothiorhodospiraceae* (Imhoff, 1984a) are halophilic phototrophic purple sulfur bacteria that, during oxidation of sulfide, deposit elemental sulfur microscopically visible outside or in the peripheral, periplasmic part of their cells. They are distinguished from the Chromatiaceae by lamellar internal membrane structures and by the dependence on saline and alkaline growth conditions (Imhoff, 1989). *Ectothiorhodospiraceae* have a characteristic composition of polar lipids (Asselineau and Trüper, 1982; Imhoff et al., 1982), fatty acids (Thiemann and Imhoff, 1996) and quinones (Imhoff, 1984b; Ventura et al., 1993), which distinguishes this group from Chromatiaceae and purple nonsulfur bacteria (Imhoff and Bias-Imhoff, 1995).

While *Halorhodospira* species are extremely halophilic bacteria and do not grow below 10% total salts, *Ectothiorhodospira* species have growth optima well below 10%. Differentiation of *Halorhodospira* and *Ectothiorhodospira* species is possible on the basis of significant differences of 16S rDNA sequences (similarities of less than 90%) and characteristic signature sequences (Imhoff and Süling, 1996; Fig. 1). In addition, fatty acid and major quinone components are different in species of both genera (Thiemann and Imhoff, 1996). *Ectothiorhodospira* species have MK-7 and either Q-7 or Q-8 as major components, whereas *Halorhodospira* species do not contain significant proportions of homologs with 7 isoprenoid units, but have MK-8 and Q-8 together with a short chain MK as major components (Imhoff 1984b; Ventura et al.,

1993). Both genera also form separate groups according to their fatty acid composition (Thiemann and Imhoff 1996; Imhoff and Süling, 1996). Properties for identification of *Ectothiorhodospira* and *Halorhodospira* species are shown in Tables 2 and 3, respectively.

For identification and verification of new isolates of Ectothiorhodospiraceae, the comparison with type strains of these bacteria deposited in and available from culture collections such as the American Type Culture Collection (ATCC in Rockville, MD) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ in Braunschweig, Germany) is necessary. The majority of types and other strains of Ectothiorhodospiraceae (and other anoxygenic phototrophic bacteria) also is available from a culture collection maintained by the author. The type strains are: *Ect. mobilis* (DSM 237), *Ect. marismortui* (DSM 4180), *Ect. shaposhnikovii* (DSM 243), *Ect. vacuolata* (ATCC 43036, DSM 2111), *Ect. haloalkaliphila* (ATCC 51935), *Ect. marina* (DSM 241), *Hlr. halophila* (DSM 244), *Hlr. halochloris* (ATCC 35916), *Hlr. abdelmalekii* (ATCC 35917), and *Thiorhodospira sibirica* (ATCC 700588).

## Preservation

For short-term preservation, well-grown cultures may be kept in closed, air-tight screw-cap bottles at 4°C in a refrigerator or at room temperature for several months, even years. Cultures of *Hlr. halochloris* and *Hlr. abdelmalekii* lose viability much faster than do cultures of other Ectothiorhodospiraceae. Strains of *Ect. mobilis* and *Hlr. halophila*, however, have been kept on a laboratory desk (room temperature and dim light) for five years and still contained numerous viable cells that grew readily after transfer into fresh medium. Maintenance transfer of liquid cultures should occur at intervals of not more than 6 months.

For long-term storage, preservation in liquid nitrogen is recommended. Well-grown cultures are supplemented with 50% dimethyl sulfoxide to give a final concentration of 5%, thoroughly mixed, dispensed in plastic ampoules, sealed and frozen in liquid nitrogen. Good viability has been demonstrated with several strains after more than 20 years.

## Physiology

All species of *Ectothiorhodospira*, *Halorhodospira* and *Thiorhodospira* grow well under anaerobic conditions in the light with reduced

sulfur compounds as photosynthetic electron donors, either photoautotrophically or photoheterotrophically with a number of organic compounds and inorganic carbonate. Some species, e.g., *Ect. shaposhnikovii* and *Ect. haloalkaliphila* grow microaerobically to aerobically in the dark. Growth factors are not required, but vitamin B<sub>12</sub> enhances growth of some strains.

During phototrophic growth with sulfide as an electron donor, sulfide is first oxidized to elemental sulfur which is then oxidized to sulfate, as has been shown for *Ect. mobilis* (see Trüper, 1978). Elemental sulfur is deposited outside the cells. Under alkaline growth conditions that are favorable for *Ectothiorhodospira* species, polysulfides are stable intermediates in sulfide oxidation. As a result, polysulfides are the first measurable oxidation products, and the medium becomes yellow-translucent at this stage. After sulfide depletion, elemental sulfur droplets are rapidly formed and the medium becomes cloudy to opaque. During further growth, cultures of red-colored species become pinkish and finally red if elemental sulfur disappears. These color changes are best observed under photoautotrophic growth conditions. Aspects of sulfur metabolism of *Ectothiorhodospira* and *Halorhodospira* species are summarized (Trüper, 1978; Trüper and Fischer, 1982).

Under autotrophic growth conditions, the fixation of carbon dioxide via the Calvin cycle is the major route of carbon assimilation in *Ect. shaposhnikovii* and high activities of ribulose-bisphosphate carboxylase have been found in this species (Firsov et al., 1974), as well as in *Ect. mobilis* (Sahl and Trüper, 1977) and *Hlr. haloalkaliphila* (Tabita and McFadden, 1972). Under photoheterotrophic growth conditions, considerable proportions of cellular carbon are derived from carbon dioxide, which is assimilated by pathways not involving the Calvin cycle. The assimilation of several organic carbon sources (such as acetate and propionate) depends on the presence of carbon dioxide and proceeds via carboxylation reactions (Firsov and Ivanovsky, 1974, 1975). Phosphoenolpyruvate carboxylase, ferredoxin-dependent pyruvate synthase, and oxoglutarate synthase were found in *Ect. shaposhnikovii* (Firsov et al., 1974); phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, and pyruvate carboxylase were found in *Ect. mobilis* (Sahl and Trüper, 1977). All enzymes of the glycolytic pathway and the tricarboxylic acid cycle, with the exception of oxoglutarate dehydrogenase, are present in *Ect. shaposhnikovii* (Krasilnikova, 1975). Cells grown on acetate had increased activities of isocitrate lyase, indicative of the function of the glyoxylic acid pathway. While poly-β-hydroxybutyric acid is the principal carbon reserve material in the absence of carbon

dioxide, in its presence, carbohydrates are preferentially formed from acetate and butyrate as well as from other carbon sources (Novikova, 1971).

## Salt adaptation

The circumscription of bacterial salt relations uses the salt concentration at which optimum growth occurs and defines nonhalophilic bacteria (below 0.1 M NaCl), brackish water bacteria (approx. 0.1–0.3 M NaCl), marine or slightly halophilic bacteria (approx. 0.4–1.0 M NaCl), moderately halophilic bacteria (approx. 1.0–2.0 M NaCl) and extremely halophilic bacteria (more than 2.0 M NaCl; Imhoff, 1993). Bacteria of each of these groups may show up with different degrees of salt tolerance. Even nonhalophilic bacteria may exhibit extreme salt tolerances and grow at very high concentration, though at a sub-optimal level. To adapt to high concentrations of salts and to cope with the high external osmotic pressure, bacteria and other unicellular microorganisms have to accumulate osmotic active molecules, so-called compatible solutes or osmotica, that are compatible with the molecular cell structures and metabolic processes (see Imhoff, 1986, 1993).

One of the most prominent properties of *Halorhodospira* species is their ability to adapt to the most extreme salt concentrations tolerated by eubacteria and to even grow in saturated salt solutions. They not only tolerate these high concentrations, but optimally thrive. They are extremely halophilic and *Halorhodospira halo-**phila* is the most halophilic eubacterium known (Imhoff, 1988a,b). To adapt to high and varying concentrations of salt, *Halorhodospira* species accumulate high concentrations of organic solutes to balance the outside osmotic pressure. By using <sup>13</sup>C-NMR techniques, glycine betaine has been found to be the main osmotically active cytoplasmic component in *Halorhodospira halo-**chloris* (Galinski and Trüper, 1982). Later, a new cyclic amino acid, 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid, called ectoine, and in addition trehalose were found as osmotica in *Hlr. halo-**chloris* (Galinski et al., 1985). Ectoine appears to be widely distributed among *Halorhodospira* species and other halophilic eubacteria in general (Severin et al., 1992).

In regard to their salt responses and the salt concentrations required for optimum growth, *Ectothiorhodospira* species are slightly halophilic or marine, and some, in particular *Ect. halo-**alkaliphila* and *Ect. marismortui*, are moderately halotolerant. Because of its extended salt tolerance, *Ect. halo-**alkaliphila* may successfully compete with *Halorhodospira* species in moder-

ately saline soda lakes. To cope with the salt and osmotic stress, *Ectothiorhodospira* species (*Ect. halo-**alkaliphila*, *Ect. marismortui*) accumulate glycine betaine, sucrose and a third component ( $\alpha$ -N-carbamoyl-L-glutamine amide) as osmotica (Imhoff, 1993; Imhoff and Riedel, unpubl. results; Oren et al., 1989; Severin et al., 1992). In contrast to *Halorhodospira* species, ectoine and trehalose have not been found in these species.

## Ecology

The phototrophic species of Ectothiorhodospiraceae are unique purple sulfur bacteria in their requirement for saline and alkaline growth conditions. Their habitats are waters and surface layers of sediments from saline and hypersaline environments with alkaline pH, that are characterized by the predominance of processes of the sulfur cycle, so-called sulfureta. These include salt and soda lakes, but also marine sediments and stagnant coastal waters of marine environments. Many of the salt and soda lakes are enriched in nutrients and organic matter and show abundant sulfate reduction in their sediments that causes production of sulfide. The capability to perform a photoautotrophic metabolism with reduced sulfur compounds as photosynthetic electron donor makes these bacteria primary producers of biomass based on metabolic products from microbial degradation processes in the anoxic and dark parts of their environments. They thrive in the anoxic part of these habitats that receive sufficient sunlight to perform photosynthesis and quite often form massive blooms that are visible as colored waters and sediments with the naked eye (Figures 7, 8, and 9).

## Applications

Like other phototrophic sulfur bacteria, *Ectothiorhodospira* species (in particular *Ect. mobilis*, *Ect. shaposhnikovii* and *Ect. vacuolata*) are suited for the removal of toxic sulfide from waste waters and principal investigations in this respect have been undertaken. Their growth requirements make possible their application under alkaline and saline conditions. The protective action of ectoine as well as glycine betaine for enzyme structure and possibly other biological structures have been analyzed and the application of these components as protective agents is discussed (e.g., Lippert and Galinski, 1992). Trehalose, ectoine and hydroxy-ectoine were the most protective against freeze-thawing and heat treatment.

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## *Oceanospirillum* and Related Genera

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### Introduction

Gram-negative, marine and/or halophilic aerobes and facultative anaerobes belong to several phylogenetic groups. Within the  $\gamma$ -subdivision of the Proteobacteria, a number of well known genera belonging to this group include *Alteromonas*, *Vibrio*, *Shewanella* and *Photobacterium*. This chapter covers many of the other, lesser known marine genera of the class  $\gamma$ -Proteobacteria, including *Alcanivorax*, *Fundibacter*, *Hahella*, *Marinobacter*, *Marinobacterium*, *Marinomonas*, *Marinospirillum*, *Microbulbifer*, *Neptunomonas*, *Oceanobacter*, *Oceanospirillum* and *Pseudospirillum*, as well as some related organisms that have not been properly classified, such as *Pseudomonas halophila* and strain 2-40. Historically, many of the species in this group have been classified with *Oceanospirillum*. Phylogenetic analyses of the 16S rRNA of these taxa suggest that, while these genera are all related, they are also fairly diverse and likely to represent a number of relatively deep phylogenetic groups (Fig. 1). Affiliated with these lesser known genera are the members of the family Halomonadaceae and the *Pseudomonas* spp. sensu stricto, both of which are discussed in other chapters. In addition, the genus *Balneatrix* is also affiliated with this group. This genus was isolated during a small outbreak of pneumonia and meningitis in humans in a spa therapy center in 1987 (Dauga et al., 1993) and grows in medium without NaCl. Although *Balneatrix* and *Pseudomonas* spp. are not primarily of marine origin, the other members of this group have only been found in marine or halophilic environments, suggesting that there is an important evolutionary distinction between freshwater and marine bacteria.

Although not a closely related group, the oceanospirilla have a monophyletic nature which is supported by several types of analyses. Except as noted below, sequence analyses of the 16S rRNA by both neighbor-joining and maximum likelihood methods support the grouping of these genera (Fig. 1). In the case of the maximum likelihood method, all branches are significantly positive ( $P < 0.01$ ). Bootstrap analyses also indi-

cate that this clade is robust, and it appears in 95% of the replicates. Similarly, the clade containing *Shewanella*, *Vibrio*, *Alteromonas* and the Enterobacteriaceae is clearly resolved. However, this topology is altered greatly when sequences from certain symbionts of marine animals are considered. For example, addition of the sequence of a cellulolytic nitrogen-fixing organism isolated from shipworms (Distel et al., 1991) or the species “*Candidatus Endobugula sertula*,” a symbiont of the larva of the bryozoan *Bugula neritina* (Haygood and Davidson, 1997), produces deep branches within the trees and causes the sequences of the Enterobacteriaceae and related families to move inside the oceanospirilla. Because these alternative topologies are only recovered in the presence of certain sequences, they appeared to be artifacts. The monophyletic nature of these marine oceanospirilla is also supported by DNA-rRNA hybridizations in which representatives of these genera form a cluster within rRNA superfamily II (De Vos et al., 1989; Pot et al., 1989). This cluster has a  $T_m(e)$  value of 68.8°C or above with members of the *Oceanospirillum* sensu stricto and also includes members of *Pseudomonas* sensu stricto and Halomonadaceae. Members of rRNA superfamily II excluded from this cluster include *Moraxella*, *Acinetobacter* and *Xanthomonas*. In addition, the Enterobacteriaceae, *Alteromonas* and related taxa were also excluded because they belonged to rRNA superfamily I.

On the basis of 16S rRNA sequence analysis, a number of phylogenetic subgroups appear to be within the oceanospirilla. *Pseudomonas* spp. sensu stricto and the family Halomonadaceae form two distinct and large clusters that are separated from each other and the oceanospirilla. *Oceanospirillum* sensu stricto also forms a closely related clade that is supported by high sequence similarities (>95.5%). Phylogenetic trees containing this clade are robust when calculated by a variety of algorithms, including parsimony and maximum likelihood, and have high bootstrap values. A group containing the genera *Marinomonas*, *Marinobacterium*, *Neptunomonas* and *Oceanobacter* is also found consistently

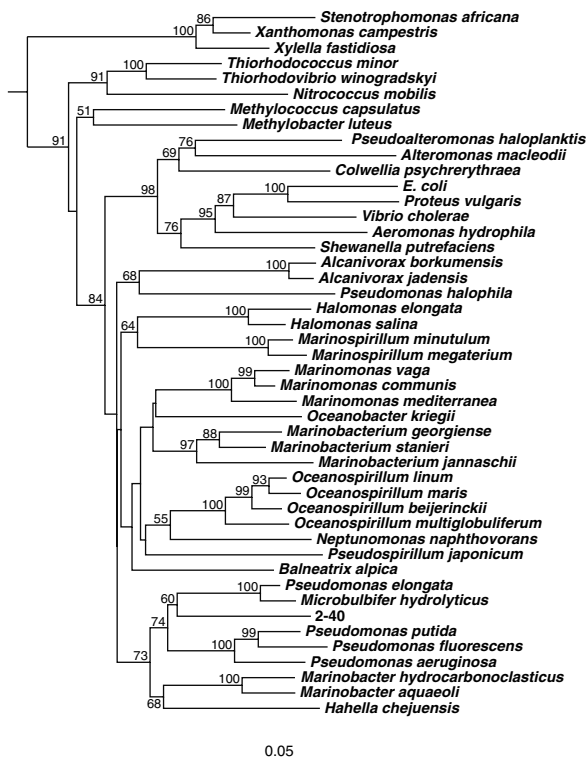


Fig. 1. Phylogeny of the marine taxa in the  $\gamma$ -subdivision of the Proteobacteria. All strains are the type strains of the species. The accession numbers for their 16S rRNA gene sequences are listed in Table 1. The accession numbers for other strains are: *Alcanivorax borkumensis*, Y12579; *Alcanivorax jadensis*, AJ001150; *Balneatrix alpica*, Y17112; *Hahella chejuensis*, AF195410; *Marinobacter aquaeoli*, AJ000726; *Marinobacter hydrocarbonoclasticus*, X67022; *Marinobacterium georgiense*, U58339; *Marinobacterium stanieri*, AB021367; *Marinomonas mediterranea*, AF063027; *Marinospirillum megaterium*, AB006770; *Microbulbifer hydrolyticus*, U58338; *Neptunomonas naphthovorans*, AF053734; *Pseudomonas elongata*, AB021368; *Pseudomonas halophila*, AB021383; and strain 2-40, AF055269. The tree was generated from the ClustalW alignment program (Thompson et al., 1994) and the neighbor-joining method using the programs in the PHYLIP package (Felsenstein, 1989). Before analysis, a filter was applied to exclude positions with less than 50% conservation within the sequences being aligned. Only positions 118 to 1396 (*Escherichia coli* numbering) were considered. *Burkholderia graminis* served as outgroup. Numbers at nodes indicate percent bootstrap values above 50 (1000 replicates). Bar indicates Jukes-Cantor evolutionary distance.

in a variety of analyses, even though the bootstrap support is modest (Fig. 1). The genus *Balneatrix* also loosely groups with these genera, although the bootstrap values are always low. Lastly, *Alcanivorax borkumensis* and *Fundibacter jadensis* are also closely related and could be classified in the same genus. In addition, the oceanospirilla contain a large number of genera that appear to represent deeply branching lineages with no close relatives in captivity:

*Hahella*, *Pseudospirillum*, *Marinobacter*, *Microbulbifer* and *Marinospirillum*. Presumably, as more oceanospirilla are isolated and characterized, additional representatives of these lineages will be found.

Marine bacteria have been the focus of increasing interest as a source of enzymes and biomaterials with technological applications. Many marine bacteria in this group were described because they degrade compounds of anthropogenic origin or produce polymers. Such has been the case for species of *Alcanivorax*, *Fundibacter*, *Hahella*, *Marinobacter*, *Marinobacterium*, *Marinomonas*, *Microbulbifer* and *Neptunomonas*. In addition, related but otherwise uncharacterized isolates also have demonstrated a large degradative capacity (Andrykovitch and Marx, 1988). Members of the genera *Alcanivorax*, *Fundibacter*, *Marinobacter* and *Neptunomonas* degrade components of petroleum. Some of these same organisms also produce emulsifiers that help to solubilize oil droplets. Members of these genera can be isolated from pristine environments, suggesting that they are a permanent component of marine communities (Bowman et al., 1997; Kaye and Baross, 2000; Golyshin et al., 2001). They are also inhabitants of oil reservoirs at both mesophilic and high temperatures and in environments with high concentrations of salts (Huu et al., 1999; Díaz et al., 2000; Kaye and Baross, 2000; Orphan et al., 2000; Golyshin et al., 2001). Since the formation of these genera, closely related isolates or 16S rRNA clones have been retrieved from petroleum contaminated sites (Ishihara et al., 1995; Harayama et al., 1999; Golyshin et al., 2001; Kasai et al., 2001), indicating an important role in degradation in nature. The ability to grow over a wide range of conditions, particularly in the presence of petroleum components (Geiselbrecht et al., 1996; Emerson and Breznak, 1997; Yakimov et al., 1998; Bruns and Berthe-Corti, 1999; Díaz et al., 2000; Orphan et al., 2000; Hedlund et al., 2001) or high concentrations of salts (Díaz et al., 2000; Kaye and Baross, 2000), has also been used to enrich for these bacteria. Currently the commercial potential of these bacteria is yet to be realized. A genomic sequence has not been performed for a member of this group, and no antibiotics or other secondary metabolites are commercially available.

In the last several years, an increasing number of genera have been added to the  $\gamma$ -3 subgroup of Proteobacteria. Other organisms with interesting characteristics have been isolated although not fully described and named. Therefore, it seems likely that many more oceanospirilla will be described in the near future. Similarly, 16S rRNA sequences of novel oceanospirilla have been retrieved directly from seawater, which suggests that these organisms are also abundant in

nature. Thus, it is likely that our understanding of this group is even more incomplete.

## The Genus *Oceanospirillum*

The genus *Oceanospirillum* was originally created to distinguish the marine from the freshwater species of the genus *Spirillum*. The genus *Spirillum* has a long history and originally contained all of the known aerobic and microaerophilic spirilla, including both marine and freshwater species (Ehrenberg, 1832; Hylemon et al., 1973). As originally defined, the genus *Spirillum* possessed species containing DNA with a much broader range of mol% G+C (38–65) and more divergent phenotypic properties than is typical for modern genera. To make the genus more consistent with other taxa, Hylemon et al. (1973) and Krieg (1984) divided it into three genera. Aerobic spirilla from freshwater habitats that could not tolerate 3% NaCl and had a DNA mol% G+C of 50–65 were placed into the genus *Aquaspirillum*. Marine strains that required seawater-based medium for growth and

possessed a DNA mol% G+C of 42–48 were placed into the genus *Oceanospirillum*. The genus *Spirillum* only retained one species, *S. volutans*, which had a DNA mol% G+C of 38 and was microaerophilic.

The genus *Oceanospirillum* described by Hylemon et al. (1973) contained five species: *O. linum*, *O. minutulum*, *O. beijeirnickii*, *O. maris* and *O. japonicum*. Terasaki (Terasaki, 1973; Terasaki, 1979) added four more species: *O. hiroschimense*, *O. pelagicum*, *O. pusillum* and *O. multiglobuliferum*; and these nine species were described in *Bergey's Manual of Systematic Bacteriology* by Krieg (1984). In addition, at about the same time, two new species, *Oceanospirillum jannaschii* and *O. kriegii*, were described (Bowditch et al., 1984). This work also transferred two species, *Alteromonas communis* and *A. vaga* to *Oceanospirillum* (Bowditch et al., 1984).

Many of these species have since been reclassified into other genera or recognized as subjective synonyms of other species. The current taxonomic status of species that have been assigned at one time or another to *Oceanospirillum* is summarized in Table 1.

Table 1. List of strains previously included in the genus *Oceanospirillum* and their current classification.

Species names	Current classification	Type strain	Accession number <sup>a</sup>	References
<i>O. beijeirnickii</i> subsp. <i>beijeirnickii</i> (Williams and Rittenberg, 1957) Hylemon et al. (1973), subsp. nov. <sup>b</sup>	<i>Oceanospirillum beijeirnickii</i>	ATCC 12754	AB006760	Williams and Rittenberg, 1957 Hylemon et al., 1973 Pot et al., 1989
<i>O. beijeirnickii</i> subsp. <i>pelagicum</i> (Terasaki, 1973) Pot et al. (1989), comb. nov. Basonym: <i>O. pelagicum</i> (Terasaki, 1973) Terasaki (1979) Other synonym: <i>Spirillum pelagicum</i> Terasaki (1973)	<i>Oceanospirillum beijeirnickii</i>	IFO 13612	AB006761	Terasaki, 1973, 1979 Pot et al., 1989
<i>O. commune</i> (Baumann et al. 1972) van Landschoot and De Ley (1983), Bowditch et al. (1984) comb. nov. Basonym: <i>Alteromonas communis</i> (Baumann et al., 1972)	<i>Marinomonas communis</i>	ATCC 27118	Ribosomal Database Project	Baumann et al., 1972 van Landschoot and De Ley, 1983 Bowditch et al., 1984 Pot et al., 1989
<i>O. hiroschimense</i> (Terasaki, 1973) Terasaki (1979) Synonym: <i>Spirillum hiroschimense</i> Terasaki (1973)	<i>Oceanospirillum maris</i>	IFO 13616	AB006762	Terasaki, 1973, 1979 Pot et al., 1989
<i>O. kriegii</i> (Bowditch et al., 1984) Satomi et al. (2001) comb. nov.	<i>Oceanobacter kriegii</i>	ATCC 27133	AB006767	Baumann et al., 1972 Bowditch et al., 1984 Satomi et al., 2001
<i>O. jannaschii</i> (Bowditch et al., 1984) Satomi et al. (2001) comb. nov.	<i>Marinobacterium jannaschii</i>	ATCC 27135	AB006765	Baumann et al., 1972 Bowditch et al., 1984 Satomi et al., 2001
<i>O. japonicum</i> (Watanabe, 1959; Hylemon et al., 1973) Satomi et al. (2001) comb. nov. Synonym: <i>Spirillum japonicum</i> Watanabe (1959)	<i>Pseudospirillum japonicum</i>	ATCC19191	AB006766	Watanabe, 1959 Hylemon et al., 1973 Satomi et al., 2001

Table 1. *Continued*

Species names	Current classification	Type strain	Accession number <sup>a</sup>	References
<i>O. linum</i> (Williams and Rittenberg, 1957) Hylemon et al. (1973) Synonym: <i>Spirillum linum</i> Williams and Rittenberg (1957)	<i>Oceanospirillum linum</i>	ATCC11336	M22365	Williams and Rittenberg, 1957 Hylemon et al., 1973
<i>O. maris</i> subsp. <i>maris</i> Hylemon et al. (1973) subsp. nov. <sup>c</sup>	<i>Oceanospirillum maris</i>	ATCC 27509	AB006771	Hylemon et al., 1973
<i>O. maris</i> subsp. <i>williamsae</i> Linn and Krieg (1978)	<i>Oceanospirillum maris</i>	ATCC 29547	AB006763	Linn and Krieg, 1978
<i>O. maris</i> subsp. <i>hiroshimense</i> (Terasaki, 1973) Pot et al. (1989) comb. nov. Basonym: <i>O. hiroshimense</i> (Terasaki, 1973) Terasaki (1979) Other synonym: <i>Spirillum hiroshimense</i> Terasaki (1973)	<i>Oceanospirillum maris</i>	IFO 13616	AB006762	Terasaki, 1973, 1979 Pot et al., 1989
<i>O. minutulum</i> (Watanabe, 1959) Hylemon et al. (1973) comb. nov. Synonym: <i>Spirillum minutulum</i> Watanabe (1959)	<i>Marinospirillum minutulum</i>	ATCC19193	AB006769	Watanabe, 1959 Hylemon et al., 1973 Satomi et al., 1998
<i>O. multiglobuliferum</i> (Terasaki, 1973) Terasaki (1979) Synonym: <i>Spirillum multiglobuliferum</i> Terasaki (1973)	<i>Oceanospirillum multiglobuliferum</i>	IFO 13614	AB006764	Terasaki, 1973, 1979
<i>O. pelagicum</i> (Terasaki, 1973) Terasaki (1979) Synonym: <i>Spirillum pelagicum</i> Terasaki (1973)	<i>Oceanospirillum beijeinckii</i>	IFO 13612	AB006761	Terasaki, 1973, 1979 Pot et al., 1989
<i>O. pusillum</i> (Terasaki, 1973; Terasaki, 1979) Satomi et al. (2001) comb. nov. Synonym: <i>Spirillum pusillum</i> Terasaki (1973)	<i>Terasakella pusillum</i>	IFO13613	AB006768	Terasaki, 1973, 1979 Satomi et al., 2001
<i>O. vagum</i> (Baumann et al., 1972) van Landschoot and De Ley (1983), Bowditch et al. (1984) comb. nov. Basonym: <i>Alteromonas vaga</i> Baumann et al. (1972)	<i>Marinomonas vaga</i>	ATCC 27119	X67025	Baumann et al., 1972 van Landschoot and De Ley, 1983 Bowditch et al., 1984 Pot et al., 1989

<sup>a</sup>16S rRNA accession number of the type strain.

<sup>b</sup>The subspecies name *O. beijeinckii* subsp. *beijeinckii* (Williams and Rittenberg, 1957) Hylemon et al. (1973) was automatically created by the valid publication of *O. beijeinckii* subsp. *pelagicum* (Terasaki, 1973) Pot et al. (1989; Rule 40d [formerly Rule 46]).

<sup>c</sup>The subspecies name *Oceanospirillum maris* subsp. *maris* Hylemon et al. (1973) was automatically created by the valid publication of *Oceanospirillum maris* subsp. *williamsae* Linn and Krieg (1978; Rule 40d [formerly Rule 46]). The subspecies *Oceanospirillum maris* subsp. *maris* Hylemon et al. (1973), appears also in Howey et al. (1990).

## Phylogeny

The phylogeny of the members of the *Oceanospirillum* core group is discussed in the introduction to this chapter.

## Taxonomy

Based only upon phenotypic characteristics, members of the genus *Oceanospirillum* were heterogeneous and difficult to classify in relation to other Gram-negative bacteria (McElroy and Krieg, 1972; Terasaki, 1972; Terasaki, 1973; Hylemon et al., 1973; Carney et al., 1975; Krieg and Hylemon, 1976). The problem was compounded by the scarcity of strains in each species. Thus, other methods have contributed greatly to our current understanding of their systematics. These include DNA-DNA hybridization (Krieg, 1984),

immunological analysis (Bowditch et al., 1984; DeLong et al., 1984), DNA-rRNA hybridization (Pot et al., 1989), 16S rRNA sequence analyses (Woese et al., 1982; Satomi et al., 1998; Satomi et al., 2001), and chemotaxonomy of polyamines, fatty acids and isoprenoid quinones (Hamana et al., 1994; Sakane and Yokota, 1994). Based upon these analyses, the classification of oceanospirilla has changed considerably. These studies demonstrated that a variety of methods, in addition to phenotypic characteristics, are necessary for the proper classification of members of the *O. linum* core group.

The *Oceanospirillum* sensu stricto or the “core group” was first recognized by Pot et al. (1989), who showed by DNA-rRNA hybridization that many species previously assigned to this genus were only distantly related to the type species *O. linum*. Instead, these species appeared to rep-

resent additional genera within the rRNA superfamily II, which also included *Oceanospirillum* sensu stricto as well as the fluorescent pseudomonads, *Deleya* (now *Halomonas*), *Azotobacter*, and an assortment of other bacterial groups. These results were confirmed and extended by Satomi et al. (2001). Thus, the placement of *Altermonas vaga* and *A. communis* in a new genus *Marinomonas* and not *Oceanospirillum* was confirmed (van Landschoot and De Ley, 1983; Bowditch et al., 1984). Similarly, the need to reclassify *O. kriegii*, *O. jannaschii*, *O. japonicum*, *O. minutulum* and *O. pusillum* into new genera was recognized (Satomi et al., 2001). Although the original interpretation differed, this taxonomy was supported by the observations of Bowditch et al. (1984) and DeLong et al. (1984), who found a close immunological relationship among superoxide dismutases and glutamine synthetases from *O. linum*, *O. beijeinckii*, *M. jannaschii*, *O. kriegii*, *M. vaga* and *M. communis* to the exclusion of species of *Alteromonas*, which belonged to the rRNA superfamily I.

With regards to subspecies, the taxonomy presented here follows the recommendations of Satomi et al. (2001). In particular, we consider the subspecies *O. maris* subsp. *williamse* and *O. maris* subsp. *hiroshimense* to be reference strains of *O. maris* and not subspecies (Satomi et al., 2001). Hence, we do not use the designation “*O. maris* subsp. *maris*” for the type strain. Similarly, we also consider the subspecies *O. beijeinckii* subsp. *pelagicum* to be a reference strain and not a subspecies (Satomi et al., 2001). However, in absence of a formal ruling by the International Committee on Systematic Bacteriology (ICSB), it is important to note that the subspecies designations are still valid, and these taxa continue to possess standing in the nomenclature. The arguments for both of these treatments are as follows. The subspecies *O. maris* subsp. *williamse* was proposed to classify a strain similar to the type strain of *O. maris* discovered in a contaminated culture of *Spirillum lunatum* (Linn and Krieg, 1978). This strain (ATCC 29547) possessed many phenotypic properties in common with the type strain of *O. maris* (ATCC 27509), but their phenotypes were not identical. Subsequent investigators confirmed the close relationship between these strains and demonstrated that both strains were closely related to *O. hiroshimense* (Pot et al., 1989). On the basis of some phenotypic differences and differences in DNA hybridization and protein patterns during gel electrophoresis, each of these strains was proposed to represent a subspecies. Similarly, *O. beijeinckii* subsp. *pelagicum* was created to recognize the similarity of *O. pelagicum* to the type strain of *O. beijeinckii* (Pot et al., 1989). Although these proposals have the advantages of maintaining the epithets, they obscure the similarities between these

strains (Satomi et al., 2001). In addition, because surveys of large collections of strains have never been performed, these subspecies have not been shown to represent genetic or phenotypic groups that might exist in nature. By assigning these strains as reference strains, the taxonomy does not prejudice the question of whether subspecies exist as biological entities. The species remaining in the *Oceanospirillum* core group are phenotypically and genotypically similar (Tables 2 and 3). They are Gram-negative spirilla, which are motile by bipolar flagellar tufts. They are heterotrophs and obligate aerobes whose major substrates are amino acids or organic acids but not sugars. They possess a DNA mol% G+C of 45–50. Spermidine is the most abundant polyamine, and the most abundant nonpolar fatty acids are C16 : 1, C16 : 0, and C18 : 1.

### Habitat

Most research on the distribution of *Oceanospirillum* spp. was performed prior to the development of molecular methods and the reorganization of its taxonomy. Therefore, it is not clear whether the earlier descriptions of morphotypes in natural environments refer to the core taxon of *Oceanospirillum* or other, morphologically similar but phylogenetically different groups (Pot et al., 1992). So far, rRNA genes closely related to *Oceanospirillum* species have not been encountered in libraries prepared from marine environmental rDNA (Giovannoni et al., 2000), which suggests that the abundance of *Oceanospirillum* in the marine environment is low. On the other hand, strains whose 16S rDNA possessed 93–95% sequence similarity to *Oceanospirillum* sensu stricto were isolated relatively frequently from seawater without prior enrichment on a low nutrient, complex medium, marine R2A agar (Suzuki et al., 1997). Therefore, the members of this taxon cannot be too rare.

What is known about the distribution of the spirillum morphotype in marine samples has been reviewed by Pot et al. (1992). Marine spirilla have been isolated from the alimentary tracts of shellfish, seawater samples taken from the intertidal zone, and decaying seaweed. The abundance in the open sea is unknown, although direct observation of low numbers of spirilla in the Gulf of Mexico has been reported (Oppenheimer and Jannasch, 1962).

### Isolation

The species of *Oceanospirillum* in the core group have been isolated from enrichments of coastal seawater and infusions of marine shellfish (Williams and Rittenberg, 1957; Hylemon et al., 1973; Terasaki, 1970; Terasaki, 1973; Terasaki, 1979). Suzuki et al. (1997) isolated organisms that were

Table 2. Helical and rod-shaped marine  $\gamma$ -Proteobacteria related to *Oceanospirillum*.

	<i>Martinospirillum</i>	<i>Oceanospirillum</i>	<i>Pseudospirillum</i>	<i>Alcanivorax</i>	<i>Hahella</i>	<i>Martinobacter</i>	<i>Martinobacterium</i>	<i>Martinomonas</i>	<i>Microbulbifer</i>	<i>Nephtomonas</i>	<i>Oceanobacter</i>
Morphology	Helical	Helical	Helical	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Number and arrangement of flagella	1, Polar or bipolar tufts	Bipolar tufts	Bipolar tufts	None	1, Polar	1, Polar or none	1, Polar	Single bipolar or 1, polar	None	1, Polar	1, Polar
Optimal temperature (°C)	15–25	25–32	30–32	20–30	20–45	30–32	37	20–25	25–37	nd	20–30
Growth at 4°C	+	d	–	–	–	–	+	–	–	+	–
Growth at 45°C	–	–	–	–	+	+	–	–	–	–	–
Optimal NaCl (%) for growth	2–3	0.5–8	nd	3–10	2	3–6	0.6–2.9	0.7–3.5	0.6–2.9	1.75–7	nd
Maximal NaCl (%) for growth	10	8	8	12.5–15	8	20	11.6	nd	6	7	nd
Nitrate reduced to nitrite	d	–	–	+	+	+	d	d	–	–	–
Nitrite reduced to N <sub>2</sub>	–	–	–	–	nd	d	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+	d	+	+	+
Catalase	– or W	d	– or W	+	+	+	+	nd	+	+	nd
Gelatin liquefaction	–	d	– or W	+	+	d	–	d	+	+	–
Starch hydrolysis	–	d	– or W	–	nd	–	–	–	+	–	–
Urease	–	–	–	–	–	d	nd	–	–	–	nd
Utilization of											
D-Glucose	–	–	–	–	+	–	+	+	+	+	+
D-Fructose	–	–	–	–	+	–	+	d	d	+	+
D-Mannose	–	–	–	–	+	–	+	d	d	–	–
Sucrose	–	–	–	–	+	–	–	–	–	–	–
Cellobiose	nd	–	nd	–	+	–	+	d	+	+	–
D-Mannitol	nd	–	nd	–	+	–	nd	+	–	–	–
Glycerol	–	–	–	–	+	–	+	+	–	+	–
Gluconate	nd	nd	nd	–	nd	–	nd	+	nd	–	–
Succinate	+	d	+	–	nd	+	+	+	+	+	nd
L-Glutamate	+	d	+	–	nd	+	+	+	+	+	d
L-Aspartate	–	–	–	–	nd	–	+	d	–	–	–
D,L-Alanine	–	–	–	–	nd	–	+	+	d	+	nd
L-Arginine	d	–	–	–	nd	–	–	d	+	+	d
L-Serine	–	–	–	–	nd	–	–	d	d	+	–
Pyruvate	+	d	+	+	nd	–	+	d	+	+	nd
Acetate	+	d	+	+	nd	+	+	+	+	+	nd
Hexadecane	nd	nd	nd	+	nd	+	nd	–	nd	nd	nd
<i>p</i> -Hydroxybenzoate	nd	d	–	–	nd	+	+	–	nd	nd	nd
PHB accumulation	+	+	+	–	nd	–	+	–	–	–	+
Mol% G+C in DNA	42–45	45–50	44–46	53–64	55	56–58	55	46–49	58	46	54–56

Symbols and abbreviations: +, present in all strains; –, lacking in all strains; d, differs among strains; nd, not determined; W, weak reaction; PHB, poly- $\beta$ -hydroxybutyrate.

Table 3. Phenotypic characteristics of members of the *Oceanospirillum* core group.

Feature	<i>O. linum</i>	<i>O. maris</i>	<i>O. beijerinckii</i>	<i>O. multiglobuliferum</i>
Cell diameter (µm)	0.2–0.6	0.6–1.1	0.6–1.2	0.5–0.9
Length of cell (µm)	3.5–30.0	2.5–40.0	2.0–15.5	2.0–10.0
Type of helix	C	C	C	C
Wavelength of helix (µm)	1.5–4.0	3.0–7.0	3.0–7.2	3.5–5.0
Helix diameter (µm)	0.7–1.5	1.2–2.8	1.0–3.0	1.0–2.0
Polar membrane present	nd	+	+	nd
Flagellar arrangement	BT	BT	BT	BT
Intracellular PHB formed	+	+	+	+
Cocoid bodies predominant after 3–4 weeks	+	+	+	+
Cocoid bodies predominant after 24–8 h	–	–	–	+
Optimum temperature (°C)	30	25	32–35	30
Temperature range (°C)	11–38	2–33	7–41	6–37
Range of NaCl (%) for growth	0.5–8.0	0.5–8.0	0.5–8.0	0.5–4.0
Nitrate reduced to nitrite	–	–	–	–
Oxidase	+	+	+	+
Catalase	+ or W	+ or W	+ or W	+
Gelatin liquefaction	W	–	d	–
Production of H <sub>2</sub> S	d	+	d	W
Phosphatase	+	d	+	+
Production of indole	–	–	–	–
Hydrolysis of casein, starch, esculin and hippurate	–	–	–	nd
Urease	–	–	–	nd
Growth in presence of 1% oxgall	+	+	+	nd
Growth in presence of 1% glycine	+	d	–	nd
Deoxyribonuclease	–	d	+	nd
Ribonuclease	d	d	+	nd
Auxotrophic growth requirement	+ <sup>a</sup>	d	–	–
Utilization of				
Glucose	–	–	–	–
Fructose	–	–	–	–
Sucrose	–	–	–	–
Xylose	–	–	–	–
Formate	–	–	–	–
Acetate	–	d	d	+
Propionate	–	d	d	+
Butyrate	–	d	d	+
Succinate	–	d	+	+
Fumarate	–	d	+	+
Malonate	–	–	–	–
Lactate	–	d	d	+
Citrate	–	–	d	+
Malate	–	d	+	+
Tartrate	–	–	–	–
Pyruvate	–	d	+	+
Methanol	–	–	–	–
Ethanol	–	d	d	–
<i>n</i> -Propanol	–	d	d	–
Glycerol	–	–	–	–
L-Alanine	–	–	–	nd
L-Glutamate	–	+	–	nd
L-Proline	–	d	–	nd
Nonpolar fatty acids (%)				
C16:1 <sup>b</sup>	47	48	48	44
C16:0	16	29	27	28
C18:1	30	13	16	20
3-Hydroxy fatty acid (%)				
C10:0 <sup>b</sup>	100	100	62	100
C14:0	0	0	30	0
Major ubiquinone	Q-8	Q-8	Q-8	Q-8
Spermidine content (µmol/g of wet wt) <sup>c</sup>	0.7	0.9	0.6	0.4
Putrescine content (µmol/g of wet wt) <sup>c</sup>	0.02	0.03	0.03	0.08
Mol% G+C in DNA	48–50 <sup>d</sup>	45–47 <sup>d</sup>	47–49 <sup>d</sup>	46 <sup>e</sup>

Symbols and abbreviations: +, present in all strains; –, lacking in all strains; d, differs among strains; nd, not determined; C, clockwise or right-handed helix; BT, bipolar tufts; W, weak reaction; and PHB, poly-β-hydroxybutyrate.

<sup>a</sup>*Oceanospirillum linum* grows poorly or not at all in defined media with single carbon sources and ammonium ions as the nitrogen source. However, abundant growth occurs in a defined medium containing succinate plus malate as carbon sources and methionine as nitrogen source.

<sup>b</sup>From Sakane and Yokota (1994). Mean values are reported.

<sup>c</sup>From Hamana et al. (1994). Mean values are reported.

<sup>d</sup>By the thermal denaturation and HPLC methods.

<sup>e</sup>By the HPLC method only.

Data from Pot et al. (1992) and also Terasaki (1972).

related to the *Oceanospirillum* core group without enrichment using a modified version of R2A medium (Reasoner and Geldreich, 1985). This medium contained in g/liter of 75% seawater: yeast extract, 0.5; proteose peptone, 0.5; casamino acids, 0.5; dextrose, 0.5; soluble starch, 0.5; sodium pyruvate, 0.3; and agar, 15. The plates were inoculated directly with 100  $\mu$ l of seawater and incubated at 15°C in the dark.

Most marine spirilla have been isolated by enrichment since they seldom predominate in the original samples. Isolation procedures included dilution in rich medium and growth in putrid infusions.

**ISOLATION BY DILUTION** Williams and Rittenberg (1957) isolated strains of *O. linum* and *O. beijerinckii* from enrichments of coastal seawater followed by a dilution technique (Krieg, 1984). Seawater was mixed with an equal volume of Giesberger's medium containing (per liter): 1 g of  $\text{NH}_4\text{Cl}$ , 0.5 g of  $\text{K}_2\text{HPO}_4$ , 0.5 g of  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , with 1% calcium lactate (Giesberger, 1936). After incubation and once spirilla appeared, a portion of the culture was autoclaved. It was then mixed with an equal volume of Giesberger's medium that lacked  $\text{NH}_4\text{Cl}$ . This medium was then inoculated with the unsterilized portion of the initial culture. Spirilla predominated after one to three subcultures in this medium. For isolation, the enrichment was diluted 1 : 100 to 1 : 100,000 in sterile seawater. After mixing, the flasks were allowed to stand for 20 minutes to allow the spirilla to migrate to the surface. Plates with the appropriate medium were then inoculated with surface water.

**ISOLATION FROM PUTRID INFUSIONS** Marine mussels and other shellfish such as *Ruditapes philippinarum*, *Crassostrea gigas*, *Batillaria multiformis* and *Mytilus edulis* were the source of strains of *O. multiglobuliferum*, *O. beijerinckii* and *O. maris* (Terasaki, 1973; Terasaki, 1979). In a typical enrichment, the bodies of one to three shellfish smashed with a hammer were submerged in a Petri dish containing 2.5% NaCl. The suspension was incubated for up to 3 days at 20–30°C. Spirilla frequently became apparent by microscopic examination early in the putrefaction. As soon as spirilla were abundant, a loopful of the suspension was removed, and the loop was touched successively to a glass slide to produce small droplets. The smallest of these droplets was then streaked on to agar medium containing peptone and 2.75% NaCl.

### Preservation

*Oceanospirilla* grow in media containing natural or synthetic seawater (Williams and Rittenberg, 1957; Hylemon et al., 1973), and isolates may be

maintained in semisolid PSS medium at 30°C with weekly transfer (Hylemon et al., 1973) or as stabs at room temperature in seawater nutrient agar with monthly transfer (Terasaki, 1972).

### Peptone-Succinate-Salt (PSS) Medium

Peptone	10 g
Succinic acid	1 g
$(\text{NH}_4)_2\text{SO}_4$	1 g
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	1 g
$\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$	0.002 g
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.002 g
Agar	1.5 g
Synthetic seawater	1 liter

The pH is adjusted to 7.8.

### Synthetic Seawater

NaCl	27.5 g
$\text{MgCl}_2$	5 g
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	2 g
$\text{CaCl}_2$	0.5 g
KCl	1 g
$\text{FeSO}_4$	0.01 g
Distilled water	1000 ml

Preservation can be accomplished by suspending a dense suspension of cells in seawater nutrient broth containing 10% (v/v) dimethyl sulfoxide, with subsequent freezing in liquid nitrogen (Krieg, 1984). Freeze-drying can be performed with cells grown on the appropriate medium (Terasaki, 1975).

### Identification

The properties of the species of *Oceanospirillum* sensu stricto are summarized in Tables 2 and 3 and below. The description of *Oceanospirillum linum* is based upon the description of *Spirillum linum* and *S. atlanticum* by Williams and Rittenberg (1957), Terasaki (1972) and Hylemon et al. (1973). The description of *Oceanospirillum maris* is based upon the description of strains 35, 36 and 37 of Hylemon et al. (1973) and *Spirillum hiroshimense* OF2 and AF1 of Terasaki (Terasaki, 1972; Terasaki, 1979). The description of *Oceanospirillum beijerinckii* is based upon the description of Hylemon et al. (1973) and *Spirillum pelagicum* UF1, AF2 and IF5 of Terasaki (Terasaki, 1972; Terasaki, 1979). The description of *Oceanospirillum multiglobuliferum* is based upon the description of Terasaki (Terasaki, 1972; Terasaki, 1979). Cells are spirilla, 0.2–1.2  $\mu$ m in diameter and 2–40  $\mu$ m in length. They form a clockwise helix, with a helical diameter of 0.7–2.8  $\mu$ m and a wavelength of 1.57  $\mu$ m. At the cell poles, a “polar membrane” underlies the cytoplasmic membrane in all species examined so far by electron microscopy. Cells also possess a Gram-negative envelope and bipolar tufts of flagella.

Cells are chemoorganotrophs and strict aerobes. Nitrate is not reduced to nitrite. Denitrifi-



cation has not been observed. Sugars are neither oxidized nor fermented. As noted in Table 3, organic acids serve as sole carbon sources for most species. Of the common amino acids, only the few shown in Table 3 are utilized. For *O. linum* and *O. beijerinckii*, amino acids, urea, nitrate, and nitrite will not serve as sole nitrogen sources. Some strains of *O. maris* utilize many but not all of the common amino acids as well as urea. *Oceanospirillum multiglobuliferum* utilizes asparagine and urea weakly, but the other amino acids have not been tested. Casein, starch, hippurate or esculin are not hydrolyzed. Growth factors are required for *O. linum* but not the other species. Cells accumulate granules of poly- $\beta$ -hydroxybutyrate (PHB). Cells are oxidase positive and indole negative.

In older cultures after 3–4 weeks, coccoid cells predominate for all species in the *Oceanospirillum* core group. In the case of *O. multiglobuliferum*, these coccoid bodies predominate after only 1–2 days. Coccoid cells are also found in older cultures of some spirilla no longer classified in the core group: *Marinospirillum minutulum* and *Terasakella pusillum*. However, *Marinobacterium jannaschii* and *Pseudospirillum japonicum* do not form these bodies. These coccoid bodies germinate in fresh medium, reforming cells with the spirillum morphology (Krieg, 1984).

Cells require seawater for growth. The range of NaCl for growth is 0.5–8.0% in medium with peptone (Terasaki, 1972; Terasaki, 1979), although they can also grow with 9.75% NaCl but not with 12.75% in PSS medium (Hylemon et al., 1973). The optimum growth temperature is 25–35°C.

The mol% G+C of the DNA ranges from 45 to 50. The type species is *Oceanospirillum linum* (Williams and Rittenberg, 1957) Hylemon, Wells, Krieg and Jannasch 1973<sup>AL</sup>. The type strain is *O. linum* ATCC 11336.

For the identification of new species, other techniques such as rRNA sequencing and DNA-DNA hybridization are recommended to supplement the phenotypic identification.

## The Genus *Marinospirillum*

The genus *Marinospirillum* was created to accommodate *Oceanospirillum minutulum*, which was originally classified as *Spirillum minutulum*, and a new isolate *Marinospirillum megaterium* (Watanabe, 1959; Satomi et al., 1998). When the genus *Spirillum* was divided into the fresh water and marine species, *M. minutulum* was moved to *Oceanospirillum* along with the other marine forms. However, a number of methods indicated that this species was only distantly related to the *Oceanospirillum* core

group. It possessed only low phenetic similarity to the type species *Oceanospirillum linum* (Terasaki, 1972; Carney et al., 1975). DNA-rRNA hybridizations also indicated that *O. linum* was no more closely related to *M. minutulum* than species from other genera in RNA superfamily II (Pot et al., 1989). Similarly, the fatty acid composition of *M. minutulum* was significantly different from that of members of the *Oceanospirillum* sensu stricto (Sakane and Yokota, 1994). Finally, it was classified on the basis of the 16S rRNA gene sequence as the type species of a new genus, *Marinospirillum*, along with the new isolate *M. megaterium* (Satomi et al., 1998).

Both species of *Marinospirillum* were isolated from environments rich in organic matter. *Marinospirillum minutulum* was isolated from a putrid infusion of a marine shellfish (Watanabe, 1959). Enrichment was carried out in synthetic medium with peptone, and the bacterium was finally isolated in medium with calcium lactate and peptone as carbon sources. Two strains of *M. megaterium* were isolated from kusaya gravy, used to make Japanese dried fish (Satomi et al., 1998). This gravy is rich in nutrients, contains 3% NaCl, volatile nitrogen compounds, and low oxygen concentrations. The strains were enriched on the basis of their high motility, which allowed them to migrate rapidly through medium in a horizontal glass tube. Following repeated enrichment in this manner, pure cultures were isolated upon streaking on Petri plates. Apparently, the ability to form colonies on Petri plates is low, and this property was lost upon subsequent transfers.

The phenotypic properties of the species of *Marinospirillum* are described in Tables 2 and 4. In addition, the spirillum of *M. minutulum* forms a clockwise spiral with a helical length of 1.5–3.0  $\mu\text{m}$  and a helical diameter of 0.8–1.3  $\mu\text{m}$  (Terasaki, 1972; Fig. 2). *Marinospirillum minutulum* could not grow with the following sole carbon sources: arabinose, galactose, lactose, maltose, mannose, saccharose, *n*-butanol, ethylene glycol, and mannite (Terasaki, 1972). Hylemon et al. (1973) further reported that this species was able to grow with 2-ketoglutarate, glutamate, glutamine and proline but not with aconitate, isocitrate, caproate, 2-hydroxybutyrate, hydroxyproline, ornithine, citrulline, putrescine, or any of the remaining common amino acids (except for tryptophan and threonine, which were not tested). As sole nitrogen sources, it utilized ammonia, asparagine and urea, but not nitrite and nitrate (Terasaki, 1972). In addition, Hylemon et al. (1973) found that it utilized alanine, aspartate, glutamine, asparagine, proline, histidine, leucine, and valine as sole nitrogen sources, but not the other amino acids tested. The major nonpolar fatty acids of *M. minutulum* are C16:0 (35%), C18:1 (32%),

Table 4. Phenotypic characteristics of the species of *Marinospirillum*.

Feature	<i>Marinospirillum minutulum</i>	<i>Marinospirillum megaterium</i>
Morphology	Helical	Helical
Cell width (µm)	0.2–0.4	0.8–1.2
Cell length (µm)	2–6	5–15
Number and arrangement of flagella	1, Polar or bipolar tufts	Bipolar tufts
Forms coccoid bodies	+	+
Temperature range (°C)	4–30	4–25
Optimal temperature (°C)	15–22	20–25
Oxygen requirement	Aerophilic	Microaerophilic
Range of NaCl for growth (%)	0.2–10.0	0.5–9.0
Optimal NaCl (%)	2–3	3
Range of pH for growth	7.0–10.5	7.5–9.0
Optimal pH	6.8–7.4	8.0
Nitrate reduced to nitrite	+	–
Nitrite reduced to N <sub>2</sub>	–	–
Oxidase	+	+
Catalase	+	– or W
Gelatin liquefaction	–	–
Growth with 1% glycine	+	–
Growth with 1% ox gall	+	–
Production of H <sub>2</sub> S	–	nd
Production of indole	–	nd
Starch hydrolysis	–	–
Urease	–	–
Phosphatase	–	–
DNase	–	–
RNase	–	–
Utilization of		
D-Glucose	–	nd
D-Fructose	–	nd
Sucrose	–	nd
Xylose	–	nd
Formate	–	nd
Acetate	+	nd
Propionate	+	nd
Butyrate	+	nd
Succinate	+	nd
Fumarate	+	nd
Malonate	–	nd
Lactate	+	nd
Citrate	–	nd
Malate	+	nd
Tartrate	–	nd
Pyruvate	+	nd
Methanol	–	nd
Ethanol	–	nd
<i>n</i> -Propanol	–	nd
Glycerol	–	nd
Ubiquinone type	Q-8	Q-8
Mol% G+C in DNA	42.5, <sup>a</sup> 42–44.1 <sup>b</sup>	44–45 <sup>a</sup>
Type strain	ATCC 19193 IFO 15450	H7 JCM 10129

Symbols: see footnote in Table 3.

<sup>a</sup>By the HPLC method.

<sup>b</sup>By the thermal denaturation method.

The data is from Watanabe (1959), Terasaki (1972), Hylemon et al. (1973), Carney et al. (1975), Sakane and Yokota (1994) and Satomi et al. (1998). For the pH, temperature, and NaCl ranges, the description of *M. minutulum* by Terasaki (1972) and Satomi et al. (1998) differed slightly. The values reported are those of the latter authors.

and C16:1 (26%; Sakane and Yokota, 1994). The major 3-hydroxy fatty acids are C12:0 (61%) and C14:1 (36%). The most abundant polyamines are spermidine (0.72 µmol/g of wet cells) and

putrescine (0.10 µmol/g of wet cells; Hamana et al., 1994).

On the basis of phenotypic tests, both Terasaki (1972) and Hylemon et al. (1973) concluded that

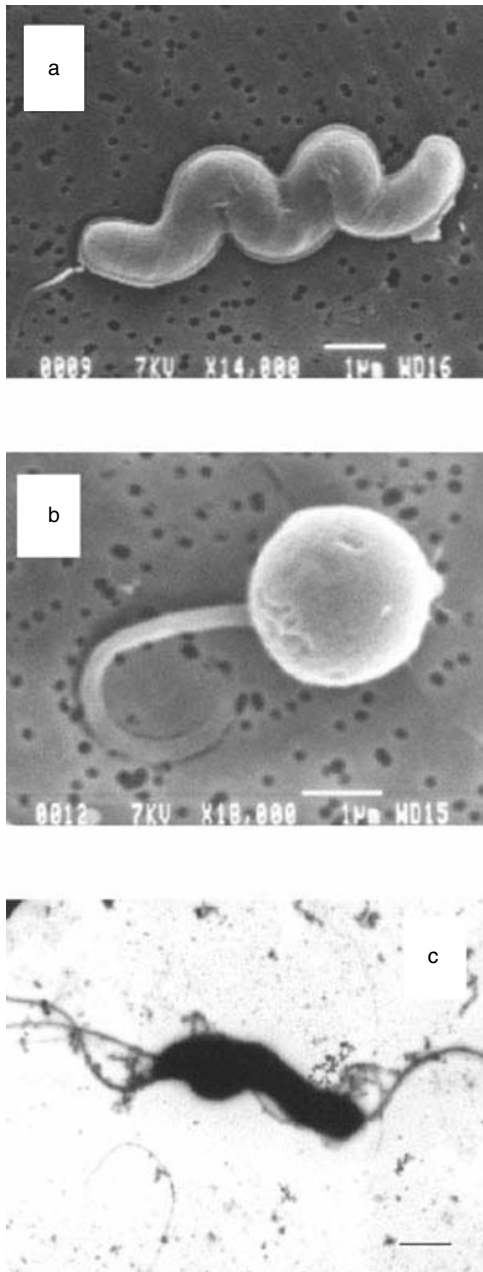


Fig. 2. Scanning electron micrographs of *Marinospirillum megaterium* H7 as vegetative cells (a), coccoid bodies (b), and negatively stained in (c). Bar is 1  $\mu\text{m}$  (a, b) and 2  $\mu\text{m}$  (c). Reproduced with permission (T. Fujii, Tokyo University of Fisheries, Japan).

strain ATCC 19192 was nearly identical to the type strain of *M. minutulum* ATCC 19193. ATCC 19192 was originally deposited as *Spirillum halophilum* (Watanabe, 1959), but the properties of the ATCC culture differed from those of the original description. For that reason, Hylemon et al. (1973) proposed that both strains be considered members of *M. minutulum*.

*Marinospirillum* shares with *Oceanospirillum* a number of characteristics. Both are Gram negative, halophilic, possess a helical shape, form coccoid bodies, and lack the ability to catabolize carbohydrates. The DNA mol% G+C content of *Marinospirillum* is slightly outside the range of the oceanospirilla (42–45 vs 45–50 for *Oceanospirillum*). Moreover, these two genera share a low level of DNA hybridization, and the sequence similarities of their 16S rRNA and *gyrB* genes are low (Pot et al., 1989; Satomi et al., 1998; Satomi et al., 2001). Phylogenetic analyses suggest that *Marinospirillum* may be phylogenetically more closely related to the Halomonadaceae than to *Oceanospirillum sensu stricto* (Fig. 1).

### The Genus *Pseudospirillum*

Watanabe (1959) isolated the type strain (ATCC 19191 or IFO15446) from putrid infusions of shellfish and described it as *Spirillum japonicum*. As with other marine spirilla, this species was enriched in seawater-based medium containing peptone as carbon source, and it was isolated in calcium lactate and peptone medium. Terasaki (Terasaki, 1963; Terasaki, 1972) described three similar strains that may belong to this or a closely related species, IF4 (IFO 15447), IF8, and UF3. These strains were all isolated from putrid infusions of shellfish at 25–30°C. The shellfish were crushed with a hammer and suspended in a 2.5% solution of NaCl in a sterile Petri plate. Microscopic observation indicated that spirilla became abundant just as the solution turned turbid. At this time, usually after 1–3 days, a small droplet was streaked onto peptone-marine salts plates for isolation.

The original classification of *S. japonicum* was based on cellular morphology. Hylemon et al. (1973) reclassified it along with the other marine strains of *Spirillum* into a new genus, *Oceanospirillum*. However, a number of independent lines of investigation indicated that this species was only distantly related to the *Oceanospirillum* core group. It possessed only low phenetic similarity to the type species *Oceanospirillum linum* (Terasaki, 1972; Carney et al., 1975). DNA-rRNA hybridizations indicated that *O. linum* was no more closely related to *P. japonicum* than species from other genera in RNA superfamily II (Pot et al., 1989). Similarly, the fatty acid composition of *P. japonicum* was significantly different from that of members of the *Oceanospirillum sensu stricto* (Sakane and Yokota, 1994). Finally, on the basis of the 16S rRNA gene sequence, it was classified as the type species of a new genus, *Pseudospirillum* (Satomi et al., 2001).

The characteristics of *P. japonicum* are described below and in Table 2. Cells of *P. japonicum* have a diameter of 0.8–1.4 µm and length of 4–15 µm. Terasaki (1972) failed to observe the helical form in the type strain, although a clockwise spiral was observed in other, closely related strains. Cells appeared as curved, straight, or flattened S-shaped rods. Longer cells, up to 75 µm in length, observed by both Watanabe (1959) and Hylemon et al. (1973), formed gentle spirals with a wavelength and helical diameter of 8–20 µm and 2–5 µm, respectively. Cells are slowly motile by means of bipolar flagellar tufts. Unlike many oceanospirilla, older cultures do not form cocci or microcysts.

*Pseudospirillum japonicum* grows under aerobic and microaerobic conditions (Terasaki, 1972). It does not respire nitrate, and it does not utilize sugars and most amino acids as sole carbon sources (Terasaki, 1972; Hylemon et al., 1973). In addition to the compounds listed in Table 3, compounds utilized include propionate, butyrate, fumarate, oxaloacetate, lactate, malate, alanine, glutamate and glutamine. None of the other common amino acids tested were utilized as sole carbon sources. Other compounds not utilized included the alcohols tested (methanol and ethanol), citrate, isocitrate and formate. In contrast, most of the common amino acids were utilized as nitrogen sources (Hylemon et al., 1973), and urea supported poor growth (Terasaki, 1972). Nitrite and nitrate were not utilized as sole nitrogen sources, and they were not reduced.

*Pseudospirillum japonicum* grows well in marine medium with 0.5–8 % NaCl (Terasaki, 1972). Growth was also obtained at initial pH values of 6.0–9.0. Higher values were not tested, and no growth was observed at pH 5.5. The temperature optimum for growth is about 35°C, and the temperature range is 11–42°C, with no growth observed at 9 and 44°C (Terasaki, 1972).

In biochemical tests, *P. japonicum* produces phosphatase, reduces selenite, and grows with 1% bile salts (Hylemon et al., 1973). It is negative for the following tests: hydrogen sulfide and indole production, casein and hippurate hydrolysis, esculin, DNase, RNase, methyl red and Voges-Proskauer, and growth with 1% glycine, triple sugar iron (TSI) agar, eosin-methylene blue (EMB) agar, and MacConkey agar.

The nonpolar major fatty acids of *P. japonicum* are C16:1 (55%), C16:0 (24%), and C18:1 (18%; Sakane and Yokota, 1994). The major 3-hydroxy fatty acid is C12:0 (97%). In addition, the most abundant ubiquinone is Q-8 (86%). The polyamines are spermidine, 1.17 µmol/g of wet weight, and putrescine, 0.01 µmol/g of wet weight (Hamana et al., 1994). The mol% G+C of the

DNA is 44.5% by liquid chromatography and 45–46% by thermal denaturation (Hylemon et al., 1973; Carney et al., 1975; Sakane and Yokota, 1994).

The sequence of the 16S rRNA gene of this species does not cluster with any other marine  $\gamma$ -Proteobacteria, and the percent similarities with described genera are all below 90%. The following characteristics define the genus: Gram-negative, curved, straight or S-shaped cells, nonsporeforming, halophilic, aerobic, chemoheterotrophic, and PHB-accumulating. Organisms do not form coccoid bodies, are motile by means of bipolar flagellar tufts, and are oxidase positive. Carbohydrates are not catabolized. The mol% G+C content of the DNA is 44–46%. The ubiquinone type is Q-8. The type species is *Pseudospirillum japonicum*. The type strain is ATCC 19191 (IFO 15446).

## The Genera *Alcanivorax* and *Fundibacter*

The members of this genus were originally described because they grow on oil-derived compounds, although they have also been isolated from pristine environments. The type species in the genus, *Alcanivorax borkumensis*, was isolated from an enrichment culture with a mixture of *n*-alkanes as the sole carbon source in seawater-based medium (Yakimov et al., 1998). The strains within this species are aerobic, Gram-negative, nonsporeforming, catalase and oxidase positive, and nonmotile rods. They catabolize a restricted spectrum of carbon sources and produce surfactants for the degradation of *n*-alkanes.

A species closely related to *Alcanivorax*, *Fundibacter jadensis*, was isolated by enrichment with hexadecane (Bruns and Berthe-Corti, 1999). Its growth requirements are very similar to those of *Alcanivorax* (Table 5) and the sequence similarity of their 16S rRNA genes is 98%. In contrast, measurements of the DNA mol% G+C contents of these two genera are far apart (53.4 and 63.6 for *A. borkumensis* and *F. jadensis*, respectively), although the differences in methods might explain the different contents. Golyshin et al. (2001) recently unified *Alcanivorax* and *Fundibacter* into one genus. Because *Alcanivorax* was the first genus described, it had priority. Thus, *Fundibacter jadensis* became *Alcanivorax jadensis*.

*Alcanivorax* strains can be isolated from seawater or marine sediments already contaminated with oil or by enrichment with hydrocarbons, either as a mixture or with a single compound (such as *n*-hexadecane). Strain MBIC 4326 was

Table 5. Phenotypic characteristics of the two species of *Alcanivorax*.

Feature	<i>Alcanivorax borkumensis</i>	<i>Alcanivorax jadensis</i> <sup>a</sup>
Morphology	Rods	Rods
Cell width (µm)	0.4–0.7	0.3–0.7
Cell length (µm)	2–3	0.8–1.8
Number and arrangement of flagella	None	None
Optimal temperature (°C)	20–30	30
Growth at 4°C	–	–
Growth at 45°C	–	–
Optimal NaCl (%) for growth	3–10	3
Maximal NaCl (%) for growth	12.5	15
Nitrate reduced to nitrite	+	+
Nitrite reduced to N <sub>2</sub>	nd	–
Oxidase	+	+
Catalase	nd	+
Arginine dihydrolase	–	nd
Lysine decarboxylase	–	nd
Ornithine decarboxylase	–	nd
Gelatine liquefaction	–	nd
Starch hydrolysis	–	nd
Urease	–	nd
Utilization of		
D-Glucose	–	–
D-Fructose	–	–
D-Mannose	–	–
Sucrose	–	–
Cellobiose	–	–
D-Mannitol	–	–
Glycerol	–	–
Gluconate	–	–
Succinate	–	–
L-Glutamate	–	–
Aspartate	–	–
DL-Alanine	–	–
L-Arginine	–	–
L-Serine	–	–
Pyruvate	+	+
Acetate	+	+
Hexadecane	+	+
Tetradecane	nd	+
Pristane	nd	+
<p>-Hydroxybenzoate</p>	–	nd
PHB accumulation	–	+
Mol% G+C in DNA	53.4 <sup>b</sup>	63.6 <sup>c</sup>
Type strain	SK2 DSM 11573	T9 DSM 12178

Symbols: see footnote in Table 3.

<sup>a</sup>Objective synonym of *Fundibacter jadensis*.

<sup>b</sup>By the HPLC method.

<sup>c</sup>By the thermal denaturation method.

Data from Yakimov et al. (1998) and Bruns and Berthe-Corti (1999).

isolated by enrichment from seawater and is able to grow on *n*-alkylcycloalkanes and *n*-alkylbenzenes (Dutta and Harayama, 2001). Harayama et al. (1999) enriched *Alcanivorax* by adding inorganic nutrients (per liter: NH<sub>4</sub>NO<sub>3</sub>, 1 g; K<sub>2</sub>HPO<sub>4</sub>, 0.2 g; and ferric citrate, 20 mg) and crude oil to pristine seawater samples. After one week of incubation at 20°C, up to 90% of the bacterial cells were estimated to be *Alcanivorax* using fluorescent in situ hybridization (FISH). Different methods have been used to detect

*Alcanivorax* spp. in seawater without cultivation. Kasai et al. (2001) detected *Alcanivorax* in both water samples and oil paste at the site of an accidental oil spill using denaturing gradient gel electrophoresis (DGGE). Members of the genus were also detected without cultivation from different marine environments (Ishihara et al., 1995; Harayama et al., 1999; Golyshin et al., 2001; Kasai et al., 2001).

The two species of *Alcanivorax* are Gram-negative rods, 0.3–0.7 µm wide and 0.8–3 µm long

(Figs. 3 and 4). In medium with alkanes as the sole carbon source, the size is larger. They are capable of anaerobic growth with nitrate and are catalase and oxidase positive. Carbon sources are limited to acetate and a few other simple organic compounds as well as aliphatic and aromatic hydrocarbons. Members of the genus are



Fig. 3. Negatively stained *Alcanivorax jadensis* T9 cells in medium with 2 ml of hexadecane per liter of modified nutrient broth. Pili are visible. Reproduced with permission (L. Berthe-Corti, University of Oldenburg, Germany).

moderately halophilic, the optimal NaCl for growth is 3–10%, and the optimum temperature is 20–30°C. Some members produce surfactants. Abundant fatty acids in the phospholipids include C16:0; sum of C16:1&969;7*cis* and C16:1&969;9*trans*; the sum of C18:1&969;7*cis*, C18:1&969;7 *trans*, C18:1&969;9*trans*, and C18:1&969;12 (these were not resolved by the chromatography system used); and an unidentified fatty acid. Colonies grown on alkane media are circular and transparent, turning opaque and light yellow following days of incubation. The type species is *Alcanivorax borkumensis* strain SK2 (DSM 11573). Phenotypically, the two *Alcanivorax* species are similar to *Marinobacter* spp., which also tolerate high concentrations of salts, do not grow on carbohydrates, and degrade hydrocarbons.

### The Genus *Hahella*

The description of this genus is based on a single strain, 96CJ10356, that produces large amounts of an emulsifying agent composed of exopolysaccharides (Lee et al., 2001). *Hahella* was isolated from marine sediments on a rich medium containing 5 g of peptone, 1 g of yeast extract, 0.01 g of FePO<sub>4</sub> in 75% aged seawater. Colonies are red pigmented.

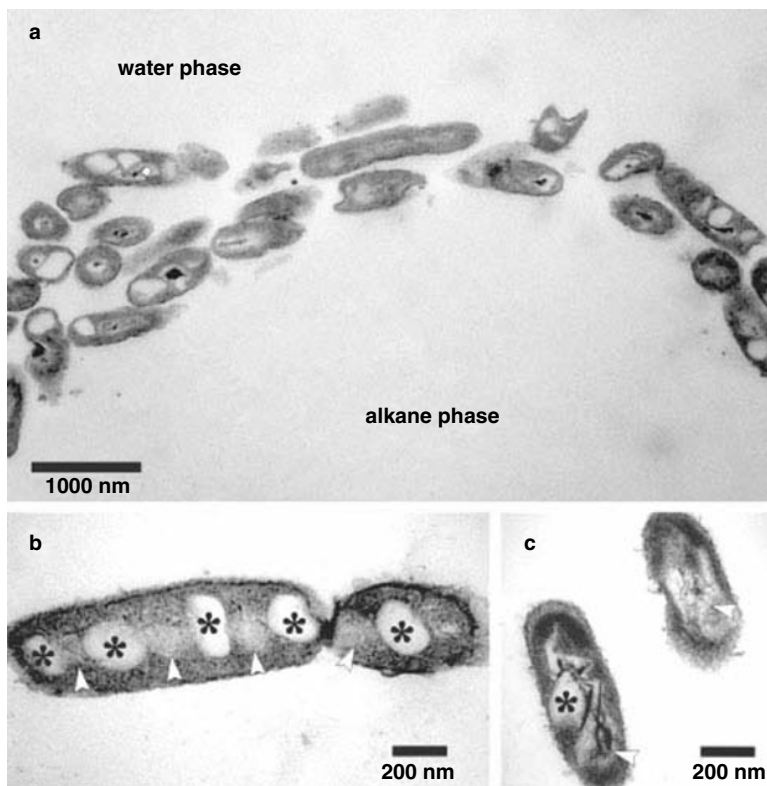
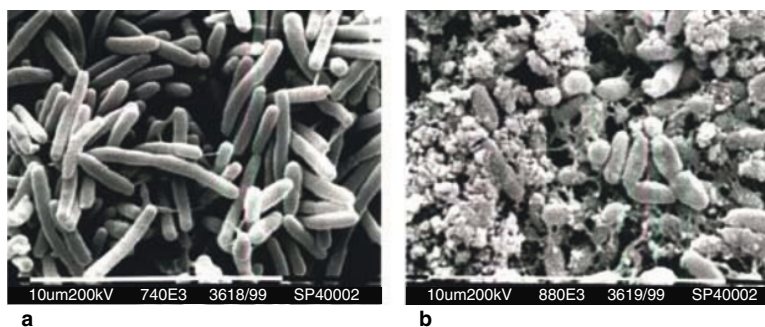


Fig. 4. Ultrathin sections of *Alcanivorax borkumensis* at different growth conditions. (a) Cells of *A. borkumensis* are growing at the water-alkane interface. Many cells show irregular morphology under these growth conditions. Characteristically electron translucent inclusions, which often dictate the outer cell shape, can be seen in cells with this physiological state. Additionally, alkane-grown cells (b) have a larger cell volume and produce more inclusions (asterisks) than (c) their pyruvate-grown counterparts. These inclusions often appear associated with the nucleoplasm (white arrowheads). Reproduced with permission (P. N. Golyshin and H. Lünsdorf, German Research Centre for Biotechnology, Braunschweig, Germany).

Fig. 5. Scanning electron micrographs of *Hahella chejuensis* in young (1-day) cultures (a). The cells become shorter in old (7-day) cultures (b). Reproduced with permission (K. S. Bae, Research Institute of Bioscience and Biotechnology, Taejon, Republic of Korea).



The following characteristics define the genus *Hahella*: the cells are Gram-negative rods each with a single polar flagellum (Fig. 5). This facultative aerobe reduces nitrate to nitrite, is oxidase and catalase positive, grows on carbohydrates with the production of acids, and hydrolyzes esculin and gelatin (Table 2). The mol% G+C of the DNA is 55 (thermal denaturation method). The type and only species is *Hahella chejuensis* strain 96CJ10356 (Korean Collection for Type Cultures number 2396). The phylogenetic classification is also unclear since only one 16S rRNA sequence exists. The percent similarity with any other sequence of the rRNA superfamily II is below 90%. Thus, *Hahella* represents a deep branch of this group.

## The Genus *Marinobacter*

The genus *Marinobacter* only contains two species, *M. hydrocarbonoclasticus* and *M. aquaeoli*. This genus is of increasing interest because many marine isolates have been characterized whose 16S rRNA sequences indicate that they could be classified in this genus. These isolates come from a variety of sources, including both pristine (Baumann et al., 1972; Pinhassi et al., 1997; Eilers et al., 2000; Hagström et al., 2000) as well as oil-contaminated environments (Gauthier et al., 1992; Emerson and Breznak, 1997; Rontani et al., 1997; Button et al., 1998; Díaz et al., 2000; Hedlund et al., 2001), hydrothermal vents (Takami et al., 1999; Kaye and Baross, 2000), sea ice (Gosink and Staley, 1995; Bowman et al., 1997), sulfide-rich sediments (Tanner et al., 2000) and oil reservoirs (Huu et al., 1999; Orphan et al., 2000). Thus, new species are likely to be described in the future that will extend the diversity of this genus.

*Marinobacter hydrocarbonoclasticus*, the first species described in this genus, was named for its ability to grow on hydrocarbons. Other strains have since been isolated from oil-contaminated environments and oil reservoirs. *Marinobacter*

*aquaeoli* was isolated from an oil-producing well (Huu et al., 1999) and was also able to grow on *n*-hexadecane, pristane and other oil components. Strains of *Marinobacter* have been also isolated from nonpolluted environments. Baumann et al. (Baumann et al., 1972; Baumann et al., 1983) described 34 strains of aerobic, oxidase-positive, Gram-negative, motile marine bacteria from pristine environments and classified them as *Pseudomonas nautica*. On the basis of its 16S rRNA gene sequence and DNA-DNA hybridization, *P. nautica* was reclassified as another strain of *M. hydrocarbonoclasticus* (Kita-Tsukamoto et al., 1993; Spröer et al., 1998).

## Phylogeny

The 16S rRNA sequences of *M. hydrocarbonoclasticus* and *M. aquaeoli* are 95.7% similar. *Marinobacter* sp. strain CAB, which was isolated for its ability to degrade isoprenoid compounds from hydrocarbon-polluted sediments with nitrate as electron acceptor (Rontani et al., 1997), was proven later to be a strain of *M. hydrocarbonoclasticus* on the basis of its 16S rRNA sequence and DNA-DNA hybridization data (Spröer et al., 1998). The 16S rRNA of the type strain of *M. hydrocarbonoclasticus*, strain CAB, and strain DSM 50418 (formerly the type strain of *Pseudomonas nautica*) possess nearly 100% sequence similarity (Spröer et al., 1998).

## Taxonomy

The characteristics of the two species in the genus *Marinobacter* are shown on Tables 2 and 6. In addition, information on the type strain of *P. nautica* was included in the description of *M. hydrocarbonoclasticus* (Baumann et al., 1972; Baumann et al., 1983). Information on the other strains was not included because, in the absence of rRNA sequences, their affiliation with this taxon is still uncertain.

Table 6. Phenotypic characteristics of the two species in the genus *Marinobacter*.

Feature	<i>Marinobacter hydrocarbonoclasticus</i>	<i>Marinobacter aquaeoli</i>
Morphology	Rods	Rods
Number and arrangement of flagella	1–3, polar or none	1, polar <sup>a</sup>
Cell width (µm)	0.3–0.6	0.4–0.5
Cell length (µm)	2–3	1.4–1.6
Temperature range	10–45	13–50
Optimal temperature (°C)	32	30
pH range	5–10	5–10
Optimal pH	7–7.5	7
Optimal NaCl (%) for growth	3–6	5
Maximal NaCl (%) for growth	20	20
Nitrate reduced to nitrite	+	+
Nitrite reduced to N <sub>2</sub>	d	–
Oxidase	+	+
Catalase	+	+
Arginine dihydrolase	–	–
Lysine decarboxylase	–	nd
Esculin hydrolysis	–	nd
Ornithine decarboxylase	–	nd
Gelatin liquefaction	–	+
Starch hydrolysis	–	nd
Urease	–	+
Phosphatase	+	+
Glucose oxidized	–	–
Glucose fermented		
Without nitrate	–	–
With nitrate	–	–
Utilization of		
D-Glucose	–	–
D-Fructose	–	–
D-Mannose	–	–
D-Arabinose	–	–
Maltose	–	nd
N-Acetylglucosamine	–	–
Sucrose	–	–
Cellobiose	–	–
D-Mannitol	–	–
Glycerol	–	–
Gluconate	–	nd
Succinate	+	+
DL-Lactate	nd	+
Citrate	+	+
Malate	+	nd
Fumarate	nd	+
L-Glutamate	+	+
L-Glutamine	nd	+
L-Aspartate	–	nd
DL-Alanine	d	+
L-Arginine	–	nd
L-Leucine	nd	+
L-Proline	nd	+
L-Serine	–	nd
Acetate	nd	+
Hexadecane	+	+
Pristane	nd	+
Phenanthrene	–	nd
<i>m</i> -Hydroxybenzoate	–	nd
<i>p</i> -Hydroxybenzoate	–	nd
PHB accumulation	–	nd
Mol% G+C in DNA <sup>b</sup>	57–58	55.7
Type strain	SP. 17 ATCC 49840	VT8 ATCC 700491

Symbols: see footnote in Table 3.

<sup>a</sup>Flagellum is frequently polar.

<sup>b</sup>By the HPLC method.

Data is from Gauthier et al. (1992), Spröer et al. (1998) and Huu et al. (1999).



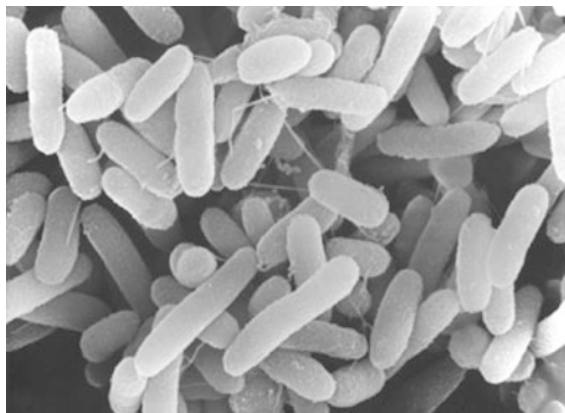


Fig. 6. Scanning electron micrograph of *Marinobacter aquaeoli* strain VT8. The cells were grown on *Halomonas* complex medium containing 5% NaCl. Reproduced with permission (H. Stan-Lotter, Institut für Genetik und Allgemeine Biologie, Salzburg, Austria; G. Wanner, Botanisches Institut der Universität München, München, Germany).

### Identification

The characteristics of the genus are as follows: Gram negative, rod-shaped, nonsporeforming, 0.3–0.6  $\mu\text{m}$  by 1.4–3.0  $\mu\text{m}$ , motile by means of a single polar flagellum (Fig. 6). Optimal growth is at 30–32°C, and the temperature range is 10–50°C. The optimal NaCl concentration for growth is 3–6%, but growth is observed in up to 20% NaCl. Growth is aerobic, nonfermentative, or anaerobic with nitrate or nitrite. Cells are oxidase and catalase positive. They utilize a few amino acids for growth but not carbohydrates. Acetate, butyrate, succinate, lactate, fumarate and citrate are also utilized as well as a range of aliphatic and aromatic compounds. Strains have been isolated from the marine habitat, especially from oil-contaminated environments. The mol% G+C of the DNA is 56–58 (HPLC method). The type species is *Marinobacter hydrocarbonoclasticus* strain ATCC 49840.

### Physiology

Members of this genus are known for their ability to degrade compounds present in oil. Fernandez-Linares et al. (1996) studied the effect of NaCl concentrations on the degradation of eicosane by the type strain of *M. hydrocarbonoclasticus*. Under different conditions, the rate of degradation of this compound did not change, and an emulsifying agent was produced for the attachment of the cells to the hydrocarbon and further degradation.

*Marinobacter hydrocarbonoclasticus* possesses vesicles or blebs made out of the outer membrane that are associated with the cells or free in the culture medium (Fernandez-Linares

et al., 1996; Gauthier et al., 1992). These vesicles may function in the uptake of nutrients from the surroundings, inasmuch as they increase the surface area of the cell. They could also be involved in the degradation of insoluble compounds. These vesicles are mostly produced when the carbon source in the medium is an insoluble compound such as eicosane. Similar vesicles have been seen in Gram-negative and Gram-positive bacteria, and in some cases, activities of hydrolytic enzymes have been found associated with the vesicles (Forsberg et al., 1981; Antranikian et al., 1987; Gauthier et al., 1992; González et al., 1997). Unidentified bacteria inside decaying wood also produce these vesicles (Daniel et al., 1987; Singh et al., 1990).

Rontani et al. (1999) describe the production of isoprenoid wax esters by all three strains of *M. hydrocarbonoclasticus* during growth on the isoprenoid compounds 6, 10, 14-trimethylpentadecan-2-one and phytol. The formation of wax esters from these types of compounds had not been previously described in bacteria and may provide an explanation for the existence of wax esters in marine or lacustrine sediments. However, the distribution of this activity in other marine bacteria is not known, so the importance of *Marinobacter* in this process remains to be demonstrated.

### Isolation

Baumann et al. (1972) enriched for *P. nautica* (*M. hydrocarbonoclasticus*) on the basis of its ability to denitrify in medium containing 0.2% sodium butyrate (Baumann et al., 1971). Presumably, strains of this genus could also be enriched in medium with the components of oil. Kaye and Baross (2000) selected for *Marinobacter* and *Halomonas* strains. Using broth containing 16% NaCl and a carbon source of 0.1% sodium citrate, they were able to grow 0.01–28% of the bacteria in various samples of seawater. Partial sequences of the 16S rRNA gene from 14 isolates indicated that they belonged to either *Marinobacter* or *Halomonas*. Thus, similar growth conditions could be used for the isolation of new *Marinobacter* strains.

*Marinobacter* strains also produce an emulsifying agent that allows them to attach to hydrocarbon particles (Fernandez-Linares et al., 1996). This characteristic might be useful for the isolation of additional species.

## The Genus *Marinobacterium*

The type species *Marinobacterium georgiense* was isolated from coastal seawater by enrichment and dilution in medium that contained filter-

sterilized seawater and low amounts of reduced nitrogen and phosphate. The carbon source was the high molecular weight fraction of pulp mill effluent, which contained mainly lignin and lower amounts of cellulose and hemicellulose (González et al., 1997). The genus also contains strains formerly classified in the genera *Oceanospirillum* and *Pseudomonas*. Satomi et al. (2001) proposed that *Oceanospirillum jannaschii* (Bowditch et al., 1984) and *Pseudomonas stanieri* (Baumann et al., 1983) represent species of *Marinobacterium*. In addition, on the basis of high DNA-DNA hybridization to *M. georgiense*, *Pseudomonas iners* (Iizuka and Komagata, 1964) was reclassified as a reference strain of this species (Satomi et al., 2001). These assignments were supported by analyses of both the 16S rRNA and *gyrB* gene sequences, as well as phenotypic characteristics and mol% G+C content of the DNA. For instance, the 3-hydroxy fatty acid profile of *P. iners* is different from that of *Pseudomonas aeruginosa* and related taxa (Oyaizu and Komagata, 1983), which supports its classification in another genus. On the basis of DNA-rRNA

hybridization data, Pot et al. (1989) also suggested that *Oceanospirillum jannaschii* had been misclassified and that this species should be included in a separate genus. Moreover, the original criteria used to classify *M. jannaschii* in the genus *Oceanospirillum* were very general and included a requirement of seawater for growth, accumulation of poly- $\beta$ -hydroxybutyrate, inability to utilize carbohydrates and a mol% G+C of the DNA within the range of 42–52%. Many organisms that are only distantly related are now known to share these characteristics.

The characteristics of the genus are as follows: Cells are rod-shaped, Gram negative, and motile by means of a single polar flagellum (Tables 2 and 7; Fig. 7). They are strict aerobes, able to grow on a wide range of substrates, including carbohydrates, organic acids, amino acids and aromatic compounds. Nitrate may be reduced. They are oxidase and catalase positive. Gelatin is not liquified. Poly- $\beta$ -hydroxybutyrate is accumulated. The mol% G+C of the DNA is 55. The type species is *Marinobacterium georgiense* strain KW-40 (ATCC 700074).

Table 7. Phenotypic characteristics of the two species in the genus *Marinobacterium*.

Feature	<i>Marinobacterium georgiense</i>	<i>Marinobacterium stanieri</i>	<i>Marinobacterium jannaschii</i>
Morphology	Rods	Rods	Rods
Number and arrangement of flagella	1, Polar	1, Polar	1–2, Polar
Cell width ( $\mu\text{m}$ )	0.5–0.7	nd	1–1.4
Cell length ( $\mu\text{m}$ )	1.6–2.3	nd	2.4–3.2
Growth at 4°C	+	–	–
Growth at 45°C	–	–	–
Nitrate reduced to nitrite	–	+	+
Nitrite reduced to N <sub>2</sub>	–	–	nd
Oxidase	+	+	+
Catalase	V	+	+
Arginine dihydrolase	nd	–	–
Gelatin liquefaction	–	–	–
Chitin hydrolysis	–	–	–
Starch hydrolysis	–	–	–
Utilization of			
D-Glucose	+	–	–
D-Fructose	+	–	–
D-Mannose	+	–	–
D-Arabinose	nd	–	–
Maltose	nd	–	–
N-Acetylglucosamine	–	–	–
Sucrose	–	–	–
Cellobiose	+	–	–
D-Mannitol	nd	–	–
Glycerol	+	–	–
Gluconate	nd	–	–
Succinate	+	+	+
DL-Lactate	nd	+	+
Citrate	+	d	d
Malate	+	d	+
Fumarate	nd	+	+
L-Glutamate	+	d	d
L-Aspartate	+	–	d

Table 7. Continued

Feature	<i>Marinobacterium georgiense</i>	<i>Marinobacterium stanieri</i>	<i>Marinobacterium jannaschii</i>
Methanol	+	nd	–
Methylamine	+	nd	nd
Ethanol	+	+	+
1-Propanol	+	+	+
1-Butanol	+	+	+
DL-Alanine	+	+	+
L-Arginine	–	d	+
L-Leucine	–	–	d
L-Proline	+	+	+
L-Serine	–	–	d
Acetate	+	+	nd
Propionate	+	+	+
Pyruvate	+	+	+
<i>n</i> -Hexadecane	nd	–	–
<i>m</i> -Hydroxybenzoate	nd	d	–
<i>p</i> -Hydroxybenzoate	+	d	–
PHB accumulation	+	+	+
Mol% G+C in DNA <sup>b</sup>	55	55	55
Type strain	KW-40 ATCC 700074	146 ATCC 27130	207 ATCC 27135

Symbols: see footnote in Table 3.

Data is from Baumann et al. (1983), Bowditch et al. (1984), González et al. (1997) and Satomi et al. (2001).

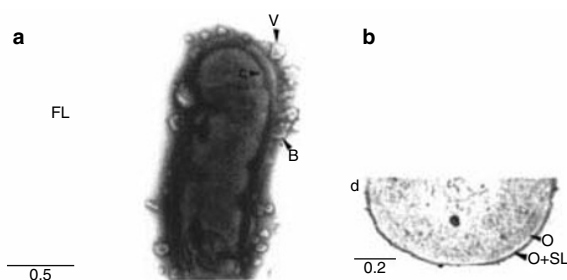


Fig. 7. (a) Negatively stained cells of *Marinobacterium georgiense* strain KW-40. The cells have a polar flagellum (FL) and the surface is covered by blebs (B) and vesicles (V). (b) Ultrathin section of strain KW-40 that shows the outer membrane (O) and S-layer (SL). C, cytoplasmic membrane. Bar indicates dimension in micrometers. Reproduced with permission (F. Mayer, University of Göttingen, Germany).

*Marinobacterium* spp. may be involved in the transformation of important sulfur compounds. Dimethylsulfoniopropionate (DMSP) is an osmolyte of marine algae and an important S-containing compound in seawater, especially in coastal environments. Dimethylsulfide (DMS) is derived from DMSP and is also an important intermediate in the global sulfur cycle. Because it is volatile, DMS plays a role in the transportation of sulfur to the atmosphere and cloud formation (Charlson et al., 1987). Ansedé et al. (2001) used a medium containing 1 mM DMSP to isolate strains of *Marinobacterium* spp. Another strain, DMS-S1, was isolated on succinate and was able to use DMS as sulfur source only in the presence of light (Fuse et al., 2000). Compounds like DMS and other organic sulfur

compounds are also produced during wastewater treatment and are of environmental concern. *Marinobacterium georgiense* was isolated from enrichment with pulp mill effluent, which also contains high molecular weight organic sulfur and aromatic compounds.

*Marinobacterium* 16S rRNA sequences were obtained from water samples associated with an oil reservoir after amplification with universal primers (Voordouw et al., 1996). Out of a total of 47 sequences analyzed, 12 belonged to the *Marinobacterium* group, which indicated that this genus was abundant in these samples and may be involved in the oxidation of sulfide that originated from sulfate reduction. The salinities in the samples ranged from 2–7% NaCl.

## The Genus *Marinomonas*

Many of the aerobic, Gram-negative heterotrophs isolated from seawater are members of the genera *Alteromonas* or *Marinomonas*. Because these genera share many phenotypic characteristics, they were discussed in the same chapter of the 2nd edition of *The Prokaryotes*. *Marinomonas* contains three species, *M. communis*, *M. vaga* and *M. mediterranea*.

The species *M. communis* and *M. vaga* were originally described as species of *Alteromonas* (Baumann et al., 1972). The genus *Alteromonas* was created to accommodate Gram-negative heterotrophs with a single polar flagellum that differed from *Pseudomonas* on the basis of their low DNA mol% G+C (38–50). In contrast, the

DNA mol% G+C of *Pseudomonas* ranged from 55 to 64 (Baumann et al., 1972). *Alteromonas communis* and *Alteromonas vaga* could be distinguished from other *Alteromonas* spp. by their ability to utilize D-sorbitol, DL-malate,  $\alpha$ -ketoglutarate and *m*- and *p*-hydroxybenzoate, and their inability to produce gelatinase or lipase (Baumann et al., 1984). In addition, they degrade aromatic compounds, whereas *Alteromonas* spp. do not (Baumann et al., 1972). They also possess bipolar flagella, as opposed to *Alteromonas* spp., which have only one polar flagellum (Baumann et al., 1984). Lastly, van Landschoot and De Ley (1983) demonstrated that *A. vaga* and *A. communis* belonged to a different DNA-rRNA hybridization group than other species of *Alteromonas* and created the genus *Marinomonas*. The type species is *M. communis*, and the type strain is *M. communis* ATCC 27118. Only a year later, Bowditch et al. (1984) independently proposed that these species be classified within *Oceanospirillum*, on the basis of immunological studies of the iron-containing superoxidase dismutases and glutamine synthetases. They found that these enzymes crossreacted most strongly with antisera prepared to the enzymes from *Oceanospirillum beijerinckii* and *Oceanospirillum jannaschii*. They also further characterized species in the genus *Oceanospirillum*, as well as 33 and 17 strains of *M. communis* and *M. vaga*, respectively. However, Pot et al. (1989) using again DNA-rRNA hybridization demonstrated that these strains were not closely related to the type species of *Oceanospirillum*, and they are currently classified in *Marinomonas*. This conclusion was later supported by 16S rRNA sequencing (Satomi et al., 1998; Satomi et al., 2001). More recently, Solano and Sanchez-Amat (1999) described a third species, *Marinomonas mediterranea*, which produces melanin.

When the genus *Marinomonas* was created, it could not be clearly differentiated from other groups of marine, Gram-negative bacteria solely on the basis of phenotypic characteristics. More-

over, while isoprenoid quinone content will distinguish *Marinomonas* from many other Gram-negative, aerobic bacteria routinely isolated from seawater, it will not distinguish species in the genera *Alteromonas*, *Pseudoalteromonas* and *Marinomonas* (Akagawa-Matsushita et al., 1992). The use of 16S rRNA sequence analysis or DNA-DNA hybridization readily resolves these taxa.

### Phylogeny

All three species in the *Marinomonas* group are phylogenetically closely related, with a percent similarity of their 16S rRNA sequences greater than 95%. *Marinobacterium* spp. and *Neptunomonas* sp. are their closest relatives, with percent similarities slightly higher than 90%. Together with *Marinomonas* and *Oceanobacter*, these five genera formed a robust cluster in rRNA phylogenetic trees when different ingroup and outgroup sequences and parsimony and maximum likelihood analyses were used (J. M. González, unpublished observations). Similarly, analysis of the *gyrB* gene of *M. vaga* and *M. communis* supports a similar conclusion (Satomi et al., 2001). Thus, in spite of its apparent phenotypic similarities, species in the genus *Marinomonas* are not closely related to *Alteromonas* or *Pseudoalteromonas* (Fig. 1).

### Taxonomy

Species of the genus *Marinomonas* were originally included in the genus *Alteromonas* because of their phenotypic similarities. When DNA-RNA hybridization data suggested that *Alteromonas* was paraphyletic, the genus *Marinomonas* was created (van Landschoot and De Ley, 1983). An additional species, *M. mediterranea*, has since been isolated, supporting the hypothesis that this genus is phylogenetically deep (Solano and Sanchez-Amat, 1999). The characteristics of the species in the genus are presented in Tables 2 and 8.

Table 8. Phenotypic characteristics of the three species of *Marinomonas*.

Feature	<i>Marinomonas communis</i>	<i>Marinomonas vaga</i>	<i>Marinomonas mediterranea</i>
Morphology	Curved rods	Straight rods	Straight rods
Number and arrangement of flagella	Single bipolar	Single bipolar	1, Polar
Cell width ( $\mu\text{m}$ )	0.7–0.9	0.8–1.1	nd
Cell length ( $\mu\text{m}$ )	1.8–2.5	2–2.8	nd
Optimal temperature			
Growth at 4°C	–	–	–
Growth at 35°C	+	+	–
Growth at 40°C	+	–	–
Growth at 45°C	–	–	–
Nitrate reduced to nitrite	–	–	+
Nitrite reduced to N <sub>2</sub>	–	–	–
Oxidase	+	–	–

Table 8. *Continued*

Feature	<i>Marinomonas communis</i>	<i>Marinomonas vaga</i>	<i>Marinomonas mediterranea</i>
Gelatin liquefaction	–	–	+
Alginase	–	–	nd
Lipase	–	–	+
Starch hydrolysis	–	–	–
Chitinase	–	–	nd
Glucose oxidized	+	+	+
Glucose fermented			
Without nitrate	–	–	–
With nitrate	–	–	–
Utilization of			
D-Glucose	+	+	+
D-Fructose	+	+	–
D-Mannose	d	+	+
Citrate	+	+	+
Aconitate	+	+	nd
Sucrose	–	–	nd
Cellobiose	–	d	nd
Malate	+	+	+
Sorbitol	+	+	+
$\alpha$ -Ketoglutarate	+	+	–
D-Mannitol	+	+	nd
Erythritol	–	+	nd
Glycerol	+	+	+
$\gamma$ -Aminobutyrate	+	+	nd
Gluconate	+	+	nd
Succinate	+	+	+
Fumarate	+	+	nd
DL-Lactate	+	+	nd
meso-Inositol	+	+	nd
L-Glutamate	+	+	nd
L-Aspartate	d	d	nd
DL-Alanine	+	+	nd
L-Ornithine	+	+	nd
L-Arginine	+	d	nd
L-Serine	+	d	nd
Pyruvate	+	+	nd
Acetate	+	+	nd
n-Hexadecane	–	–	nd
N-Acetylglucosamine	–	d	nd
m-Hydroxybenzoate	+	+	–
p-Hydroxybenzoate	+	+	nd
Sarcosine	+	+	nd
Putrescine	+	d	nd
Betaine	+	–	nd
Pigmentation	–	–	+
PHB accumulation	–	–	–
Mol% G + C in DNA	46–48 <sup>a</sup>	46–48 <sup>a</sup>	46 <sup>b</sup>
Type strain	8 ATCC 27118	40 ATCC 27119	MMB-1 ATCC 700492

Symbols: see footnote in Table 3.

<sup>a</sup>By the buoyant density method.

<sup>b</sup>By the HPLC method.

Data from Baumann et al. (1972) and Solano and Sanchez-Amat (1999).

## Habitat

*Marinomonas* can be isolated from seawater. *Marinomonas communis* and *M. vaga* were isolated from open ocean seawater samples taken in the Hawaiian archipelago by Baumann et al. (1972). *Marinomonas mediterranea* and a number of uncharacterized *Marinomonas* strains

(Ansele et al., 2001) were isolated from coastal seawater.

## Isolation

*Marinomonas communis* and *M. vaga* can be enriched by amending 500 ml of seawater with 25 ml of a solution of 1 M Tris-HCl (pH 7.5), 0.5

g of  $\text{NH}_4\text{Cl}$ , 38 mg of  $\text{K}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$ , 14 mg of  $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ , and 0.5 g of *m*-hydroxybenzoate. The culture is incubated at 20–25°C for up to 10 days. Isolates are obtained on Basal Medium Agar (BMA) plates containing 0.1% *m*-hydroxybenzoate (Baumann et al., 1984).

#### BMA

Tris-HCl (pH 7.5)	6.1 (or 12.1) g
$\text{NH}_4\text{Cl}$	1.0 g
$\text{K}_2\text{HPO}_4 \cdot 3 \text{H}_2\text{O}$	75 mg
$\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$	28 mg
Distilled water	500 ml
Artificial seawater	500 ml

#### Artificial seawater (ASW)

NaCl	23.4 g
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	24.6 g
KCl	1.5 g
$\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	2.9 g
Distilled water	1000 ml

Using a complex medium, Eilers et al. (2000) found that 6% of the isolates from a seawater sample taken directly from the North Sea were strains of *Marinomonas*. However, prefiltration through a 1.2  $\mu\text{m}$  pore size filter favored the isolation of *Marinomonas*, and five out of nine isolates obtained under these conditions were close relatives of *M. communis*.

Additional isolates of *Marinomonas* spp. were obtained by Ansele et al. (2001) using media containing 1 mM of the osmolyte dimethylsulfoniopropionate (DMSP). Either basal salts or f/2 media were used.

#### Basal Salts Medium

NaCl	2.34 g
$(\text{NH}_4)_2\text{SO}_4$	0.66 g
$\text{MgSO}_4$	0.096 g
$\text{CaCl}_2$	0.0333 g
Fe EDTA	3.27 mg

The pH is adjusted to 7.2 by adding potassium phosphate to a final concentration of 10 mM. Serial dilutions of seawater or sediment slurries are plated directly on this medium. In some cases, bacteria are first enriched in f/2 medium (Guillard, 1975) with 1 mM DMSP prior to plating.

#### Identification

*Alteromonas* and *Marinomonas* are phenotypically similar. *Marinomonas communis* and *M. vaga* may be distinguished from *Alteromonas* by their ability to utilize D-sorbitol, DL-malate,  $\alpha$ -ketoglutarate, and *m*- and *p*-hydroxybenzoate, and inability to produce gelatinase or lipase. *Marinomonas mediterranea*, however, is unable to grow on *m*-hydroxybenzoate and has gelatinase and lipase activities.

#### Physiology

*Marinomonas communis* and *M. vaga* grow on D-fructose and D-glucose using the Entner-Doudoroff pathway. This pathway may be widespread among members of the large group of marine  $\gamma$ -Proteobacteria (Baumann and Baumann, 1973; Sawyer et al., 1977).

#### Applications

*Marinomonas mediterranea* is melanogenic (Solano et al., 1997; Solano and Sanchez-Amat, 1999; Solano et al., 2000) and produces polyphenol oxidase, an enzyme involved in melanin synthesis. These enzymes are commonly isolated from fungi and are of interest because of their potential biotechnological applications in polymerization of phenols, oxidation of xenobiotics, pulp bleaching, and oxidation of lignin substrates. *Marinomonas mediterranea* strain MMB-1 is one of the few bacterial isolates where laccase activity (one of the enzymes of the family of polyphenol oxidases) has been detected. Although not closely related, strain 2-40 has also been found to have polyphenol oxidase.

#### The Genus *Microbulbifer*

The genus *Microbulbifer* only contains one species, *M. hydrolyticus*, which was isolated by enrichment from coastal seawater (González et al., 1997). In addition, *Pseudomonas elongata* is closely related and should be reclassified into this genus (Anzai et al., 2000). The following properties are for both *M. hydrolyticus* and *P. elongata* and are characteristic of the genus. Cells are narrow Gram-negative rods, nonmotile, strictly aerobic, and oxidase and catalase positive (Tables 2 and 9). The temperature range for growth is 10–41°C, with an optimum at 25–37°C. Seawater-based medium is required for growth; the optimum NaCl concentration is 0.06–3.0%. Carbon sources utilized include carbohydrates, some amino acids and simple aromatic compounds such as vanillate and ferulate. Agar, cellulose, xylan, chitin, gelatin, starch and Tween 80 are hydrolyzed. Synthetic lignin is mineralized to a limited extent. The mol% G+C of the DNA is 58. The type species is *M. hydrolyticus* strain IRE-31 (ATCC 700072).

The cells possess vesicles or blebs derived from the outer membrane that may be an adaptation for degradation of polymeric compounds. These vesicles are attached to the cells or free in the culture medium and may function for the uptake of nutrients from the surroundings or involved in the degradation of insoluble compounds (Fig. 8). Similar vesicles have been

Table 9. Phenotypic characteristics of *Microbulbifer hydrolyticus* and the related species *Pseudomonas elongata*.

Feature	<i>Microbulbifer hydrolyticus</i>	<i>Pseudomonas elongata</i>
Morphology	Rods	Rods or coccoid bodies
Cell arrangement	Single or long chains	Single or long chains
Cell width (µm)	0.3–0.5	0.3–0.4
Cell length (µm)	1.1–1.7	3–6
Number and arrangement of flagella	None	nd
Optimal temperature (°C)	37	25–30
Growth at 4°C	–	nd
Growth at 45°C	–	nd
Optimal NaCl (%) for growth	0.6–2.9	2.0–3.0
Maximal NaCl (%) for growth	5.8	6
Nitrate reduced to nitrite	–	–
Nitrite reduced to N <sub>2</sub>	–	nd
Oxidase	+	nd
Catalase	+	+
Gelatin liquefaction	+	+
Chitin hydrolysis	+	+
Cellulose hydrolysis	+	+
Agar liquefaction	–	+
Starch hydrolysis	+	+
Urease	nd	–
Glucose oxidized	+	+
Glucose fermented		
Without nitrate	–	–
With nitrate	–	–
Utilization of		
D-Glucose	+	+
D-Fructose	–	+
D-Mannose	–	+
Sucrose	–	nd
Cellobiose	+	nd
D-Mannitol	nd	–
Glycerol	–	–
Lactate	nd	+
Citrate	–	–
Propionate	+	+
Succinate	+	+
L-Glutamate	+	+
Aspartate	–	–
DL-Alanine	+	–
L-Arginine	+	+
L-Serine	+	–
Pyruvate	+	nd
Acetate	+	+
<p>-Hydroxybenzoate</p>	–	nd
PHB accumulation	–	nd
Mol% G+C in DNA	57.6 <sup>a</sup>	nd
Type strain	IRE-31 ATCC 700072	ATCC 10144

Symbols: see footnote in Table 3.

<sup>a</sup>By the HPLC method.

Data from González et al. (1997) and Humm (1946).

observed in both Gram-negative and Gram-positive bacteria (Forsberg et al., 1981; Antranikian et al., 1987; Gauthier et al., 1992; González et al., 1997; González and Weiner, 2000). In some cases, hydrolytic enzymes are associated with the vesicles (Forsberg et al., 1981). Unidentified bacteria inside decaying wood also produce these vesicles (Daniel et al., 1987; Singh et al., 1990).

*Pseudomonas elongata* and *M. hydrolyticus* share a number of traits (Table 9), including the ability to degrade polymeric compounds. *Pseudomonas elongata* was isolated because of its ability to degrade agar (Humm, 1946). *Microbulbifer hydrolyticus* does not degrade agar, but both organisms degrade other polymers including cellulose, chitin, starch and gelatin.

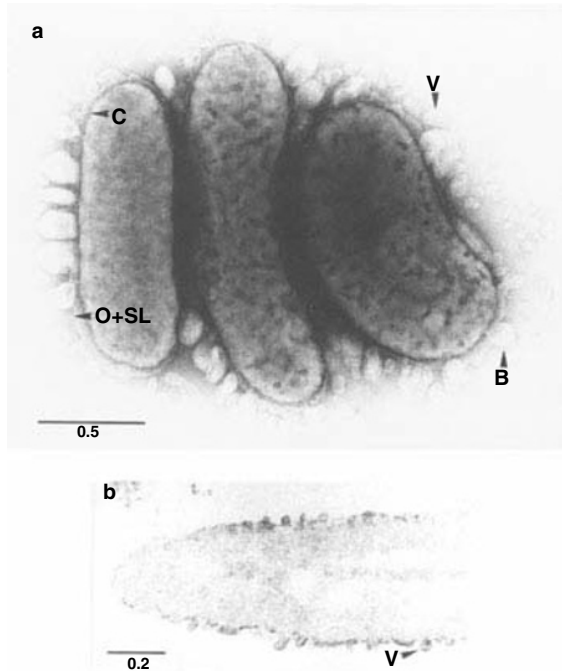


Fig. 8. (a) Negatively stained cells of *Microbulbifer hydrolyticus* strain IRE-31. The surface is covered by blebs (B) and vesicles (V) formed by extensions of the outer membrane (O) and S-layer (SL). (b) Ultrathin section of strain IRE-31 also showing vesicles (V). Reproduced with permission (F. Mayer, University of Göttingen, Germany).

They also produce exopolysaccharides and form long chains in broth, depending upon the growth conditions. Both strains were isolated from coastal seawater in environments where the salinity varies throughout the year and which receive a continuous load of plant-derived detritus.

In the case of the isolation of *M. hydrolyticus*, enrichment was carried out using lignin-rich byproducts of the pulp and paper industry. This organism dominated the enrichment after two weeks. Thus, polymeric waste products are suggested for the isolation of members of the genus from coastal seawater.

## The Genus *Neptunomonas*

The first strains of this genus were isolated from heavily contaminated sediments by using naphthalene or phenanthrene as sole carbon sources (Geiselbrecht et al., 1996). The isolates were obtained after dilution (1:20, 000), suggesting that they were abundant in the sediment.

*Neptunomonas* is one of the few genera that have been isolated from the marine environment because of their ability to grow on poly-

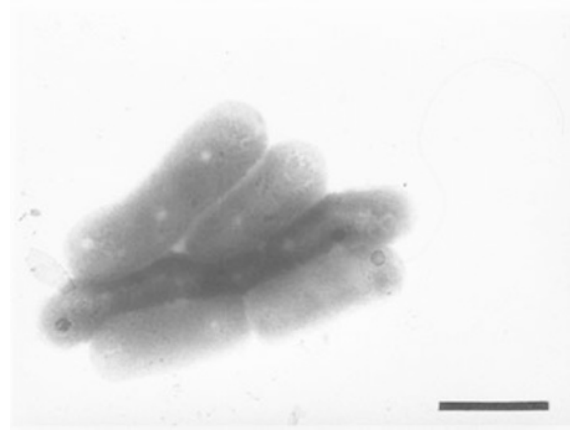


Fig. 9. Negatively stained cells of *Neptunomonas naphthovorans* NAG-2N-126. Bar is 2 μm. Reproduced with permission (J. T. Staley, University of Washington, and B. Hedlund, Universität Regensburg, Germany).

cyclic aromatic hydrocarbons (PAHs). The two characterized strains also grow on 2-methylnaphthalene or phenanthrene as the sole carbon source. The putative PAH dioxygenase genes from these two strains have also been sequenced, and they are more closely related to naphthalene and nitroaromatic dioxygenases from *Pseudomonas*, *Cycloclasticus* and *Burkholderia* than to biphenyl or monoaromatic dioxygenases (Hedlund et al., 1999).

Phylogenetic analysis of the 16S rRNA sequences groups this genus close to *Marinobacterium* spp., with similarities higher than 91%. *Neptunomonas*, *Marinomonas*, *Marinobacterium* and *Oceanobacter* also form a cluster with relatively high bootstrap values (Fig. 1).

The cells of *Neptunomonas* are Gram-negative, rods, motile by a single polar flagellum (Table 2; Fig. 9). They are facultatively aerobic, oxidase and catalase positive, able to grow on amino acids, carbohydrates, organic acids, and a few PAHs as the sole carbon source. They require sodium for growth. The mol% G+C of the DNA is 46 (thermal denaturation method). The type species is *Neptunomonas naphthovorans* strain NAG-2N-126 (ATCC 700637). The characteristics that distinguish *Neptunomonas* from phylogenetically closely related genera are listed in Table 2.

There is also some evidence that *Neptunomonas* spp. may be important in pristine environments. Clones of 16S rRNA genes related to *Neptunomonas* were retrieved without cultivation from microbial communities associated with sea ice (Brown and Bowman, 2001). The diversity of species in this genus remains to be determined because so few strains have been characterized.



## The Genus *Oceanobacter*

Strains of this genus include the “H-1 group” of Baumann et al. (1972). Although cells are rod-shaped, they were originally classified in the genus *Oceanospirillum* as *O. kriegii* on the basis of immunological analysis of their superoxide dismutases and glutamine synthetases (Bowditch et al., 1984). Subsequently, DNA-rRNA hybridizations between this species and representatives of closely related organisms as well as sequence analysis of the 16S rRNA gene supported the reclassification to a new genus, *Oceanobacter* (Pot et al., 1989; Satomi et al., 2001). Consistent with this classification, members of this species grow on some carbohydrates (D-glucose, D-fructose and mannitol), whereas members of the *Oceanospirillum* sensu stricto do not. The mol% G+C of the DNA is also outside the range of *Oceanospirillum* sensu stricto. In addition, analysis of the 16S rRNA and *gyrB* gene sequences suggests a distant relationship between the genus *Oceanobacter* and the genera *Marinomonas*, *Marinobacterium* and *Neptunomonas* (Fig. 1).

The description of the genus is as follows: Gram-negative, straight rod, nonsporeforming, halophilic, aerobic, chemoheterotrophic, PHB-accumulating and oxidase-positive bacteria (Table 2). Motility is by means of flagella. Some carbohydrates are catabolized. In addition, some strains utilize simple alcohols and organic acids, such as ethanol and lactate, as well as betaine and aminobutyrate as sole carbon sources. These bacteria reduce nitrate to nitrite. The mol% G+C content of its DNA is 54–56. The ubiquinone type is Q-8 (Sakane and Yokota, 1994). The major nonpolar fatty acids in the phospholipids are C16:1 (36 %), C18:1 (27 %) and C16:0 (16 %; Sakane and Yokota, 1994). The major 3-hydroxy fatty acids are C12:0 (54 %), C16:0 (27 %) and C10:0 (19 %). Spermidine (0.84  $\mu\text{mol/g}$  of wet cells) and putrescine (0.03  $\mu\text{mol/g}$  of wet cells) were the only detectable polyamines (Hamana et al., 1994). The type species is *Oceanobacter kriegii* strain 197 (ATCC 27133). The differential characteristics of this genus with respect to other genera are shown in Table 2.

### *Pseudomonas halophila*

*Pseudomonas halophila* was isolated from the highest dilutions of water samples taken from Great Salt Lake, Utah, that showed growth in media with acetate, glucose or glycerol (Fendrich, 1988). On the basis of the sequence of its 16S rRNA gene, *P. halophila* is distantly related to *Marinobacter* spp. (approximately 90% sequence similarity, Fig. 1). Even though the mol% G+C of the DNA is similar to that of

*Marinobacter*, the phenotype is substantially different. Thus, the 16S rRNA sequence similarity and differences in phenotypic characteristics suggest that *P. halophila* should be reclassified into a new genus.

*Pseudomonas halophila* is a Gram-negative, pleomorphic rod and motile by means of a single, polar flagellum. Cells are 0.8–1.0  $\mu\text{m}$  wide by 1.5–5.0  $\mu\text{m}$  long. It is a strict aerobe that uses a number of carbohydrates, organic acids and amino acids as carbon sources. It is catalase, oxidase and urease positive. It liquefies gelatin, and it is negative for arginine dehydrolase and for lysine and ornithine decarboxylase. Growth occurs between 0.12 and 19% NaCl, and the optimum concentration of NaCl is 5%. The temperature range for growth is 4–37°C, with an optimum at 28°C. The pH range for growth is 4.5–9.6 with an optimum growth at 7.0. The mol% G+C of the DNA is 57.

### Strain 2-40 and the Shipworm Symbionts

Strain 2-40 (ATCC 43961) was isolated from the salt marsh because it can degrade agar and produce melanin pigments (Andrykovitch and Marx, 1988). On the basis of its 16S rRNA sequence, strain 2-40 is the most closely related to nitrogen-fixing symbionts that have been isolated from shipworms (Distel et al., 1991; Sipe et al., 2000), although the percent similarities are low (91–94%). These shipworms rely on the symbionts for the degradation of cellulose present in wood and for the acquisition of reduced nitrogen (Waterbury et al., 1983). The 16S rRNA gene sequence of strain 2-40 possesses 94% similarity to that of strain TBTC, isolated from the woodworm *Bankia setacea* (Sipe et al., 2000). These strains are also related to four strains from different genera of shipworms that also possessed similar phenotypic characteristics as well as identical 16S rRNA gene sequences (Waterbury et al., 1983; Distel et al., 1991). The percent similarities with these strains is >91%. Other symbionts from marine animals have also been found whose phylogeny remains to be determined (Haygood and Davidson, 1997).

The phylogenetic affinity of these strains to other genera is not clear since they appear equally related to the genera *Pseudomonas*, *Microbulbifer*, *Oceanospirillum* and *Marinobacter*. Strain 2-40 is a Gram-negative, pleomorphic rod, and motile by means of a single polar flagellum. The average cell width is 0.5  $\mu\text{m}$  and the length is 1.5–3.0  $\mu\text{m}$ , although in stressed cultures, filaments and coils are formed. It is a strict aerobe and catalase and oxidase positive. The temperature range for growth is 4–37°C. The

pH range for growth is 4.5–10. The sea salt concentration for growth is from 1–10%, with an optimum at 2.3–3.5%. The mol% G+C of the DNA is 47 (thermal denaturation method). Strain 2-40 is able to hydrolyze a number of polysaccharides, including agar. Strain 2-40 expresses polyphenol oxidase activity involved in the production of melanins during the late phase of growth. Like the enzyme from *M. mediterranea*, the enzyme from strain 2-40 has properties of tyrosinase and laccase (Solano and Sanchez-Amat, 1999). Although the tyrosinases have a wide distribution in different organisms, laccases are only found in fungi and higher plants and are involved in the polymerization and degradation of lignin. Thus, these enzymes have potential biotechnological application for the production of melanin pigments and in lignin degradation.

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## *Serpens flexibilis*: An Unusually Flexible Bacterium

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### Habitats

Many eutrophic aquatic environments as typified by ponds or sewage lagoons harbor a wide variety of aerobic bacteria in the upper water layers. Thin, flexible bacteria such as spirilla, spirochetes, and *Serpens flexibilis* constitute a small fraction of the total microorganisms in these environments. The use of enrichment procedures to isolate these flexible bacteria usually is not successful because they are rapidly overgrown by the other bacteria, even when low-nutrient-containing media are used. Presumably, these flexible bacteria can compete in their natural environments because of factors such as high cell-surface to cell-volume ratios that allow for maximizing transport of nutrients, possession of chemotaxis mechanisms, and unusual motility properties that allow for movement through viscous solutions (Greenberg and Canale-Parola, 1977).

### Isolation

#### Selective isolation

*S. flexibilis* can be selectively isolated by using procedures originally developed for isolation of thin spirilla and spirochetes (Canale-Parola et al., 1966). A small amount of pond water or mud slurry is placed in the center of a sterile cellulose filter disc (0.3 to 0.45  $\mu\text{m}$  pore diameter) which has been placed on the surface of a petri dish containing isolation medium made with 1.0% agar. The disc is removed after aerobic incubation of the plate at 30°C for 6 to 12 h. After subsequent incubation of the plate for 2 to 4 days, a subsurface, whitish veil of growth develops. The organism can then be isolated by picking from the outer edge of the veil and streaking onto a second plate. Restreaking several times may be necessary to obtain pure, cloned cultures. An isolation medium that has been routinely used (Hespell, 1977, 1984) includes: yeast extract, 0.2 g; peptone, 0.1 g; 10 ml hay extract;

and 90 ml distilled water. The pH of the medium is adjusted to pH 7.0 with KOH prior to autoclaving. The hay extract is prepared by boiling 1.0 g of dried wheat or barley straw in 100 ml of distilled water for 15 min., cooling the mixture to room temperature, and decanting the fluid which is then clarified by centrifugation (8,000  $\times$  g, 10 min).

#### Cultivation

Optimal growth yields ( $10^9$  to  $10^{10}$  cells/ml) and growth rates (20 to 30 min doubling times) of *S. flexibilis* can be obtained in a nutrient-rich medium such as LYPP medium (Hespell, 1977): 60% sodium lactate syrup, 1.0 ml; yeast extract, 0.3 g; peptone, 0.2 g; 0.2 M potassium phosphate buffer (pH 7.4), 10 ml; and 90 ml distilled water. Typical cultures consist of 250 ml of medium in 1-liter Erlenmeyer flasks shaken at 150 to 250 rpm at 30°C. The organism also can be grown on chemically defined media such as LCH, but the growth yields and growth rates are considerably lower. LCH medium consists of: 60% sodium lactate syrup, 1.0 ml; 0.4 g ammonium chloride; trace minerals (Hespell and Canale-Parola, 1970), 1.0 ml; 0.2 M potassium phosphate buffer (pH 7.4), 20 ml; and 78 ml of distilled water. Replacement of the ammonium chloride with an equivalent amount of peptone or Trypticase markedly stimulates growth. *S. flexibilis* will not grow on media having an initial pH of 5.8 or less, and optimal growth occurs when the initial pH is 6.8 to 7.2. During growth the pH of the medium rises to 9 to 11, and increased cell yields can be obtained by periodically lowering the pH by additions of weak HCl. Highest growth yields are obtained at 28° to 33°C, but no growth occurs at 10° or at 45°C.

#### Preservation of Cultures

Long-term storage of *S. flexibilis* can be accomplished by routine lyophilization of cultures. Alternatively, cultures can be stored in liquid nitrogen or ultracold (–75 to –85°C) freezers (Hespell and Canale-Parola, 1970).

## Identification

*S. flexibilis* forms a subsurface veil of growth similar to that commonly observed with spirochetes. Microscopic examination of agar pieces removed from the leading edge of the veil show extremely flexible rods measuring about 0.3 by 10  $\mu\text{m}$  long.

Often several cells are seen together and appear as “a pit of microbial snakes.” The cells display furious lashing motions and often tend to twist in and out of knot formations [Fig. 1a](#). In liquid media, the movement is less dramatic. The long, slender cells usually move in straight lines displaying an overall flexing of the cell with the

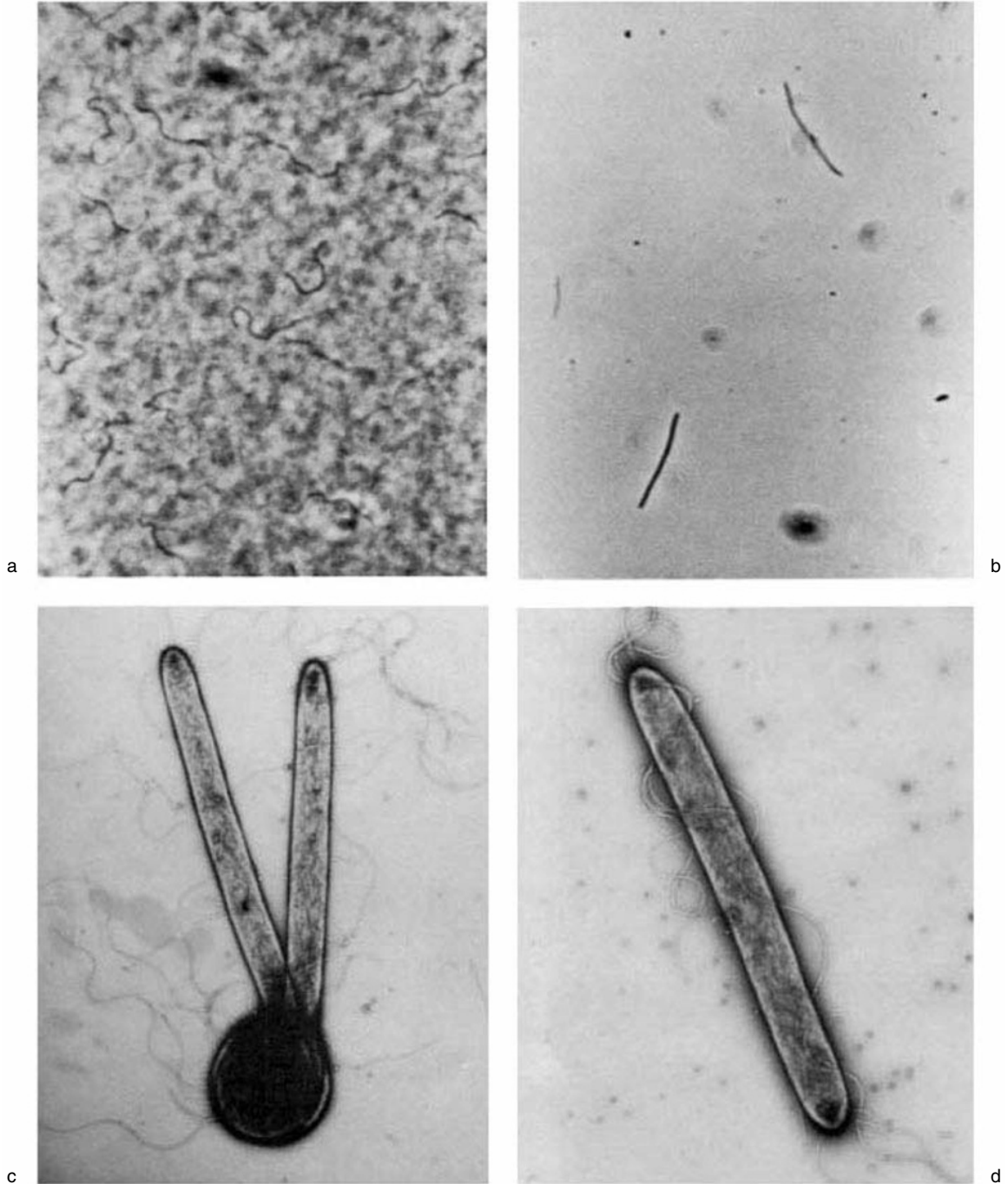


Fig. 1. Phase contrast micrographs ([Fig. 1a](#) and [b](#)) and transmission electron micrographs ([Fig. 1c](#) and [d](#)) of *S. flexibilis* strain PFR-1. Cells from subsurface growth in agar media show rapid coiling movements ([1a](#)), whereas in liquid media ([1b](#)), cells display straight-line movement of flexing rods. Cells are quite flexible and are able to coil up ([1c](#)). They are motile by means of lateral flagella ([1c](#)) and polar tufts of flagella ([1d](#)). Cells are about 0.3  $\mu\text{m}$  in diameter and 10  $\mu\text{m}$  in length.

trailing cell tip having a vibrating motion Fig. 1b. No tumbling type of movement is observed. In stationary phase cultures, clumps of entwined cells are common, and often partially lysed cells, spherical cell bodies, are present Fig. 1c. Electron microscopy shows that cells typically possess a cluster of 4 to 10 flagella that are present at the tips of both cell ends; in addition, the presence of several lateral flagella randomly distributed along the cell body is not uncommon. Fig. 1d. The flagella are quite long (15 to 30  $\mu\text{m}$ ), have a constant waveform with a 0.3  $\mu\text{m}$  amplitude, and possess the hook end and disk structure commonly observed with other bacteria.

In addition to its unusual motility characteristics, another key feature of *S. flexibilis* is the rather limited range of substrates that support its growth. Essentially, only lactate is catabolized and supports good growth. Limited growth can be obtained with  $\alpha$ -ketoglutarate, acetate, or pyruvate. In the presence of peptone, the addition of glucose and a few other disaccharides results in about a doubling of cell yields over that observed in the absence of added substrates ( $2.0$  to  $6.0 \times 10^7$  cells/ml).

Colonies can be formed under aerobic conditions on media containing 2% agar. These are usually 3 to 4 mm in diameter, off-white to cream colored, and round with filamentous edges. The cells from colonies tend to be thicker (0.5 to 1.0  $\mu\text{m}$ ) and shorter (4 to 6  $\mu\text{m}$ ). Cell pellets from liquid cultures often are light pink to reddish in color, probably due to the presence of the cytochrome proteins; the pigments cannot be extracted with chloroform, methanol, or other solvents used to remove carotenoids. Whether taken from liquid cultures or colonies, cells are always Gram negative. Electron microscopy of thin sections of *S. flexibilis* shows that the cells have a classical double-track, Gram-negative cell structure (Hespell, 1977, 1984).

The phylogenetic status of *S. flexibilis* has been examined with the 16S rRNA oligonucleotide-cataloging technique. The results indicated that *S. flexibilis* has an  $S_{AB}$  of 0.9 with *Pseudomonas pseudoalcaligenes* (Woese et al., 1982). Woese et al. (1982) concluded that *S. flexibilis* was a close relative to *P. pseudoalcaligenes* and that it might be a variant pseudomonad that developed a defect in septum formation because *S. flexibilis* forms long cells with multiple cytoplasmic invaginations (Hespell, 1977, 1984). This 16S RNA phylogenetic placement of *S. flexibilis* has been confirmed by analysis of aromatic amino acid biosynthesis enzymes of *S. flexibilis* (Ahmad and Jensen, 1987). These studies indicate this organism belongs with subgroup Ia pseudomonads. While *S. flexibilis* shares a number of common features with *P. pseudoalcaligenes*, there are a number of distinct differences. Glucose and

many other sugars do not support the growth of either organism, and both organisms can grow with lactate, acetate, or pyruvate as energy sources. However, *S. flexibilis* does not grow with succinate, citrate, ethanol, fructose, or glycerol. The GC content of the DNA of *S. flexibilis* is 66 mol% (Hespell, 1977) and that of *P. pseudoalcaligenes* strains is 62 to 64 mol% (Palleroni, 1984). *S. flexibilis* differs from many *Pseudomonas* species in having multiple flagella and in lacking poly-B-hydroxybutyrate formation. At present, it would seem reasonable to consider *S. flexibilis* to be related to the pseudomonads. Because of its distinct features, this organism should probably be classified in its own genus (*Serpens*) and species (*flexibilis*) within the family Pseudomonadaceae. Although *S. flexibilis* strains have been isolated from several geographical locations (Hespell, 1977), detailed studies have not been done to determine whether enough differences exist to justify multiple species of *Serpens*.

## Physiological Properties

*S. flexibilis* is a strictly aerobic bacterium and cannot grow anaerobically even in the presence of electron acceptors such as nitrate or nitrite. The organism displays an endogenous respiratory quotient ( $\text{CO}_2$  released  $\text{O}_2$  consumed) of 0.7 to 0.8, and this value increases to 1.56 in the presence of sodium lactate. Approximately 60% of the lactate catabolized is converted to carbon dioxide and about 30% is incorporated into cell material (Hespell, 1977). Growth on lactate-containing media results in production of trace amounts of formate and acetate. Cell extracts contain high levels of lactic acid dehydrogenase and enzyme activities associated with the tricarboxylic acid cycle. However, only trace levels of enzymes of the Embden-Meyerhof and hexose monophosphate pathways are present. Analysis of the peptidoglycan layer indicates that diaminopimelic acid is the main cross-linking compound, and the other constituents include alanine, glutamate, muramic acid, and glucosamine (Hespell, 1977). The peptidoglycan appears to be typical of that found in most Gram-negative bacteria.

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# The Genus *Psychrobacter*

JOHN P. BOWMAN

## Introduction

The genus *Psychrobacter* includes bacteria which are Gram negative, spherical to rod-shaped, strictly aerobic, chemoheterotrophic, nonmotile, cold-adapted and osmotolerant. *Psychrobacter* species are primarily isolated from cold to warm, slightly to highly saline ecosystems ranging from glacial ice to sea-ice to chilled meat and fish to clinical samples (Table 1). The genus has only recently become better known following recent intensive bacterial isolation studies of various environments accompanied by 16S rRNA gene sequencing. From these studies it is clear that *Psychrobacter* is a widespread and evolutionarily successful group of bacteria, the biology of which may provide important insights into environmental adaptation and survival.

## Taxonomy and Phylogeny

The genus *Psychrobacter* was originally described by Juni and Heym (1986) and included at that time one species, *Psychrobacter immobilis*. *Psychrobacter immobilis* strains were identified by a simple genetic transformation plate assay in which DNA isolated from strains were used to transform hypoxanthine-requiring or oxidase-negative mutants (Juni and Heym, 1980). Only DNA from *Psychrobacter* strains successfully transformed genetically closely related test strains that as a result regained the indicated phenotype. DNA from other taxa had no effect. The test was useful in sorting out the taxonomy of the large number of psychrotolerant *Achromobacter*-like and *Moraxella*-like strains isolated from fresh, frozen and irradiated food products, including fish (Georgala, 1958; Chai, 1981), poultry (Thornley, 1967; Lahellec et al., 1975), meat (McLean et al., 1951; Tiwari et al., 1972; Gill et al., 1977), some clinical samples (Bøvre, 1984; see Pathogenicity section), and including contaminants from cold-stored laboratory media.

Subsequently, rRNA-based analyses indicated the genus *Psychrobacter* belonged to the family

Moraxellaceae, which also includes the genera *Acinetobacter* and *Moraxella* (Rossau et al., 1991; Enright et al., 1994). The family Moraxellaceae forms a distinct branch in the class Gammaproteobacteria (Garrity and Holt, 2001) and is most closely related to other aerobic chemoheterotrophic members of the Gammaproteobacteria including the families Halomonadaceae, Pseudomonadaceae and Oceanospirillaceae. The family does not include genus *Neisseria* (or its relatives *Kingella* and *Eikenella*), which has some morphological and phenotypic similarities with *Moraxella*. Instead, *Neisseria* and its fellow genera belong to class Betaproteobacteria and are thus completely unrelated to the Moraxellaceae.

The genus *Psychrobacter* has rapidly acquired species in the last few years, mostly from the increasing exploration of marine and polar ecosystems. The valid species as of 2003 included *P. immobilis* as the type species (Juni and Helm, 1986; for additional data, see Bowman et al., 1996), *P. faecalis* (Kämpfer et al., 2002), *P. fozii* (Bozal et al., 2003), *P. frigidicola* (Bowman et al., 1996), *P. glacincola* (Bowman et al., 1997a), *P. jeotgali* (Yoon et al., 2003), *P. luti* (Bozal et al., 2003), *P. marincola* (Romanenko et al., 2002), *P. okhotskensis* (Yumoto et al., 2003), *P. pacificensis* (Maruyama et al., 2000), *P. phenylpyruvicus* (Henriksen and Bøvre, 1968; Bøvre, 1984; Bowman et al., 1996), *P. proteolyticus* (Denner et al., 2001), *P. pulmonis* (Vela et al., 2003), *P. submarinus* (Romanenko et al., 2002) and *P. urartivorans* (Bowman et al., 1996; Table 1). The phylogenetic arrangement of these species is shown in Fig. 1. Some species occur in pairs, e.g., *P. submarinus* and *P. marincola*, possessing very similar 16S rRNA gene sequences. Therefore, 16S rRNA gene sequences can evidently only provide a preliminary assignment of species identity within the genus; phenotypic and other genotypic data are always necessary for reliable identification (see Identification section).

The species *P. phenylpyruvicus* was previously named “*Moraxella phenylpyruvica*” (Henriksen and Bøvre, 1968; Bøvre, 1984) and included isolates obtained from a variety of clinical sources.

Table 1. Habitats, suitable growth media, and incubation temperatures for routine cultivation.<sup>a</sup>

Species	Known habitats	Media	Growth temperature (°C)
<i>P. glacincola</i>	Sea ice, and deep ice cores	MA	20
<i>P. immobilis</i>	Fish, chilled meat and blood products, ornithogenic soil, sea ice, cold seawater, and contaminants on lab media	Nutrient agar, TSA, and MA	25–30
<i>P. urativorans</i>	Antarctic ornithogenic soil, and chilled meat	TSA, and MA	20
<i>P. frigidicola</i>	Antarctic ornithogenic soil	MA	15
<i>P. proteolyticus</i>	Antarctic krill stomach	MA, and TSA + 2.6% NaCl	20–25
<i>P. phenylpyruvicus</i>	Internal organs, blood, cerebrospinal fluid of humans and various animals, and chilled meat and fish	BHI, nutrient agar + 5% horse serum, and Columbia blood agar	30–37
<i>P. faecalis</i>	Bioaerosol of pigeon feces	Nutrient agar, and MacConkeys agar	25–30
<i>P. luti</i>	Glacial mud, and seawater	TSA, and MA	25
<i>P. fozii</i>	Coastal marine sediment, and seawater	TSA, and MA	ND
<i>P. pulmonis</i>	Lungs of lambs	Columbia blood agar	30
<i>P. submarinus</i>	Seawater	MA	25
<i>P. marincola</i>	Internal tissues of ascidian ( <i>Polysyncraton</i> sp.)	MA	25
<i>P. jeotgali</i>	Jeotgal, traditional Korean fermented seafood	MA	30
<i>P. pacifiensis</i>	Seawater, and Japan Trench	MA, and nutrient agar	25–30
<i>P. okhotskensis</i>	Icy seawater	MA	25

Abbreviations: MA, marine agar; TSA, trypticase soy agar; BHI, brain heart infusion; ND, not determined.

<sup>a</sup>For all validly described *Psychrobacter* species.

Results of 16S rRNA sequence analysis and general phenotypic traits indicated the species clearly belonged to *Psychrobacter* (Bowman et al., 1996). Suggestions for a new genus for *P. phenylpyruvicus* were proposed but never carried out (Petterson et al., 1998) and in any case would be inappropriate inasmuch as the species is clearly embedded in a central phylogenetic position in the genus (Fig. 1).

## Identification

In comparison to *Psychrobacter* (which is generally psychrotolerant, osmotolerant and nutritionally non-exacting), *Moraxella* and *Acinetobacter* are mesophilic, unable to grow at 4°C and are not halotolerant or halophilic. Neither of these genera occurs in marine habitats (Table 2). The genus *Moraxella* also has quite fastidious growth requirements and is generally only isolated from animal tissues and fluids where it exists most of the time in a benign parasitic relationship. *Acinetobacter* species on the other hand are widespread in soils, freshwater and wastewater ecosystems in which *Psychrobacter* is at best rare.

## Phenotypic Characteristics

*Psychrobacter* species form cream or off-white, smooth, circular, convex colonies with a smooth margin and a buttery consistency. Some *Psychrobacter* isolates can be occasionally pale pink, pos-

sibly owing to accumulated cytochrome proteins (Bowman et al., 1997b). When grown under optimal conditions (see Growth and Isolation section), cells for all species are coccobacilli (typically 0.4–1.8 µm long and 0.4–1.6 µm wide). Juni and Heym (1986) described some strain differences including rod-like cells in chains, with the rods having either rounded or pointed ends. The cells are classified as nonmotile though they may possess numerous short fimbriae and move by “twitching” motility, as is typical for *Moraxella* and *Acinetobacter*. *Psychrobacter* species (also like other members of the family Moraxellaceae) do not form resting bodies (cysts, spores etc.), appendages, or other notable cellular architectural features. The cells though Gram negative can retain the crystal violet dye better than normal and so occasionally will stain Gram positive. Cells will however lyse rapidly in the presence of detergents (2% sodium dodecyl sulfate [SDS; w/v]) or strong alkaline solutions (3% KOH [w/v]) unlike most Gram-positive cells.

*Psychrobacter* species are distinctive as they can grow well at low temperature and can tolerate a wide range of salt concentrations (Table 3). Some species require or are strongly stimulated by sodium ions though most grow well without sodium. The variation in temperature requirements and ability to tolerate salt and ox bile salts are useful characteristics for distinguishing *Psychrobacter* species (Table 3). *Psychrobacter* species are neutrophilic growing best at pH 6.0–8.0 but not below pH 5.5 or above pH 9.0.

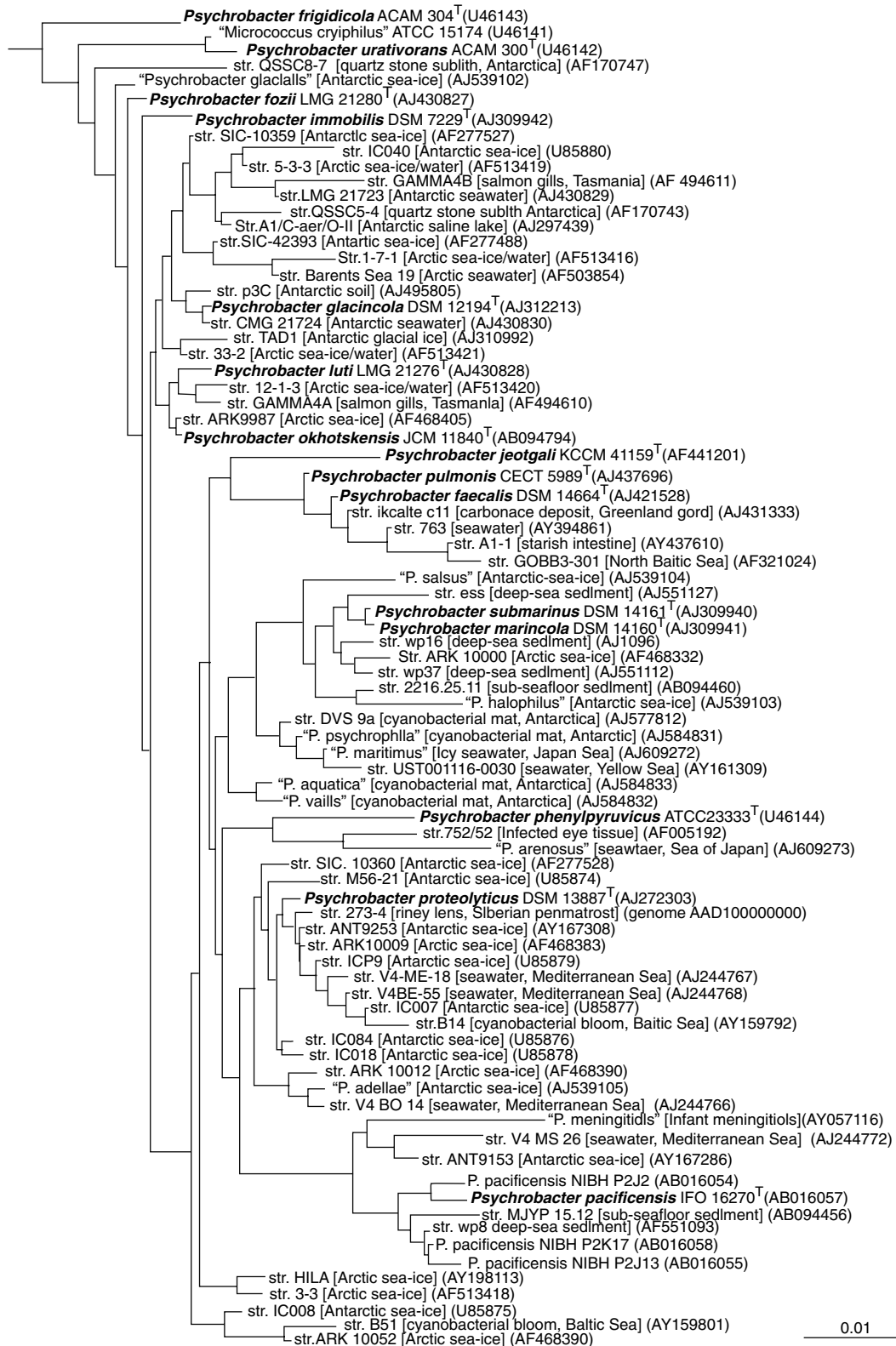


Fig. 1. A phylogenetic tree based on near complete and partial 16S rRNA gene sequences of the genus *Psychrobacter*. Branch lengths are based on maximum likelihood distances and the tree was constructed by the neighbor-joining method. Sequences of the type strains of validly named species are indicated in bold type. Names in quotation marks (e.g., "Psychrobacter glacialis" etc.) are as yet undescribed invalid species and in many cases may become new valid species of the genus in the next one to two years. Sequences indicated by "str." are either bona fide isolates or clones from published studies of the ecosystem indicated in brackets. The number in parentheses is the GenBank accession code for the given sequence. The scale bar at the base of the tree indicates a relative maximum likelihood distance of 0.01.

Table 2. Characteristics useful for differentiating *Psychrobacter* from its closest relatives *Moraxella* and *Acinetobacter*.

Trait	<i>Psychrobacter</i>	<i>Moraxella</i>	<i>Acinetobacter</i>
Oxidase test	+	+	–
Growth at 4°C	+	–	–
Growth in the presence of 6% NaCl	+	–	–
Complex growth requirements	V <sup>a</sup>	+ <sup>a</sup>	–
Occurrence in marine habitats	+	–	–

Symbols and abbreviations: +, trait is positive for most strains; V, test varies between strains or between species; and –, trait is negative for most strains.

<sup>a</sup>Some *Psychrobacter* strains require yeast extract or horse serum for good growth including some but not all strains of *P. jeotgali*, *P. phenylpyruvicus* and *P. pulmonis*. *Moraxella osloensis* does not have complex growth requirements.

Table 3. Temperature and salt tolerance of *Psychrobacter* species.

Species	Growth temperature <sup>a</sup> (°C)				Salinity requirement and tolerance (% NaCl)						Tolerance to 5% ox bile salts
	4	25	30	37	0	1–6	8	10	12	15	
<i>P. immobilis</i>	+	+	+	V/w	+	+	+	+	V/w	–	+
<i>P. faecalis</i>	+	+	+	+	+	+	+	+	–	–	+
<i>P. fozii</i>	+	+	+	–	–	+	+	+	–	–	–
<i>P. frigidicola</i>	+	–	–	–	w	+	+	–	–	–	–
<i>P. glacincola</i>	+	+	–	–	V/w	+	+	+	V/w	–	+
<i>P. jeotgali</i>	+	+	+	–	+	+	+	+	–	–	ND
<i>P. luti</i>	+	+	+	–	+	+	+	–	–	–	–
<i>P. marincola</i>	w	+	+	–	–	+	+	+	+	+	ND
<i>P. okhotskensis</i>	+	+	+	–	+	+	+	+	–	–	ND
<i>P. pacifiensis</i>	+	+	+	–	V/w	+	–	–	–	–	ND
<i>P. phenylpyruvicus</i>	+	+	+	+	+	+	+	–	–	–	+ <sup>b</sup>
<i>P. proteolyticus</i>	+	+	+	–	+	+	+	+	–	–	–
<i>P. pulmonis</i>	ND	+	+	+	+	+	ND	ND	ND	ND	–
<i>P. submarinus</i>	+	+	+	–	–	+	+	+	+	+	ND
<i>P. urativorans</i>	+	+	V/w	–	+	+	+	–	–	–	–

Symbols and abbreviations: +, most strains produce significant growth; V, trait is variable between strains; w, growth is weak (or not equivocal) for most strains; –, most strains do not grow; and ND, no data available.

<sup>a</sup>All *Psychrobacter* strains grow well between 7–20°C.

<sup>b</sup>Growth is stimulated by ox bile.

*Psychrobacter* species are always strictly aerobic showing no anaerobic growth under any condition and are almost always strongly catalase and cytochrome *c* oxidase positive. Tables 3 and 4 list some biochemical and nutritional characteristics that are useful for distinguishing valid *Psychrobacter* species. In general, *Psychrobacter* is relatively biochemically inert and will be unreactive in most of the popularly used commercial rapid tests (e.g., 20E API and 20NE API test strips, Vitek-Biomérieux). *Psychrobacter* species in general do not break down complex substrates such as polysaccharides; a minority may hydrolyze proteins (Denner et al., 2001; Table 4). Preferred carbon sources are organic acids and amino acids. Carbohydrates and other types of compounds are in general not utilized. *Psychrobacter* species often produce lipases and can break down a few substrates common in nature but not normally catabolized by most Gram-negative bacteria. One substrate is uric acid

(Bowman et al., 1996), which is the major nitrogenous end product of the metabolism of birds (high concentration in guano) and fish. Uric acid is derived from purine metabolism via hypoxanthine and xanthine and is broken down by the enzyme uricase oxidase to allantoin, which in turn is hydrolyzed to metabolites (3-phosphoglycerate and methylureate) that feed into the tricarboxylic acid cycle. Uric acid can serve as the sole source of carbon, nitrogen and energy for many *Psychrobacter* species (Bowman et al., 1996).

## Chemotaxonomy

The major fatty acids in *Psychrobacter* include the monounsaturated lipids 16:1 $\omega$ 7c (palmiteic acid), 17:1 $\omega$ 8c (heptadecenoic acid) and 18:1 $\omega$ 9c (oleic acid). Oleic acid is particularly important (Table 5) and can be used as a determinant for

Table 4. Biochemical characteristics and DNA base composition of *Psychrobacter* species.

Species	Acid from carbohydrates	Nitrate reduction	Urease	Tween 80 hydrolysis	Protease activity	Phenylalanine deaminase	Mol% G+C
<i>P. faecalis</i>	–	ND	–	ND	ND	ND	ND
<i>P. fozii</i>	–	–	+	–	–	–	45
<i>P. frigidicola</i>	–	–	–	–	–	+	41–42
<i>P. glacincola</i>	–	V	V	+	–	–	43–44
<i>P. immobilis</i>	+	V	V	+	–	+	44–47
<i>P. jeotgali</i>	–	+	+	+	–	ND	43–44
<i>P. luti</i>	–	+	–	+	+	+	44–46
<i>P. marincola</i>	–	–	–	+	–	–	50–51
<i>P. okhotskensis</i>	–	+	–	+	–	ND	47
<i>P. pacifiensis</i>	+	–	+	–	–	–	43–44
<i>P. phenylpyruvicus</i>	–	+	V	+	–	+	42–44
<i>P. proteolyticus</i>	+	–	+	w	+	ND	44
<i>P. pulmonis</i>	–	+	–	ND	ND	ND	ND
<i>P. submarinus</i>	+	–	–	+	–	–	47
<i>P. urativorans</i>	–	V	V	–	–	–	44–46

Symbols and abbreviations: +, most strains produce significant growth; V, trait is variable between strains; w, growth is weak (or not equivocal) for most strains; –, most strains do not grow; and ND, no data available.

Table 5. Major whole cell fatty acids of *Psychrobacter* species.

Species	10:0	12:0	16:1 $\omega$ 7c	16:0	i17:0	17:1 $\omega$ 8c	18:2	18:1 $\omega$ 9c	18:1 $\omega$ 7c	18:0	3-OH 12:0
	Fatty acid content %: <sup>a</sup>										
<i>P. faecalis</i>	3	4	9	4	5	6	ND	54	ND	6	6
<i>P. fozii</i>	6	ND	21	2	1	13	ND	41	ND	1	7
<i>P. frigidicola</i>	ND	ND	37–45	0–2	ND	3–5	ND	49–56	0–2	0–2	t <sup>b</sup>
<i>P. glacincola</i>	ND	T	24	2	1	1	ND	57	ND	2	4
<i>P. immobilis</i>	ND	ND	31–39	1–3	1–4	5–8	ND	46–55	t	0–2	t
<i>P. luti</i>	2	ND	16	1	1	10	ND	60	ND	2	2
<i>P. marincola</i> <sup>c</sup>	3	4	3	ND	ND	ND	ND	84	ND	ND	3
<i>P. okhotskensis</i> <sup>c</sup>	3	2	9	ND	ND	21	ND	57	2	ND	ND
<i>P. pacifiensis</i>	t–1	1–2	7–16	6–9	t–1	1–5	2–3	50–53	1	6–13	4
<i>P. phenylpyruvicus</i>	ND	ND	33	6	t	6	8	43	t	3	2
<i>P. proteolytica</i>	ND	3	23	5	t	7	ND	45	1	3	5
<i>P. submarinus</i> <sup>c</sup>	3	7	4	ND	ND	ND	ND	79	ND	ND	2
<i>P. urativorans</i>	ND	ND	40–53	2–5	0–2	5–10	t	30–45	1–3	0–1	t
<i>P. jeotgali</i>	2	3	6	1	2	9	ND	66	ND	1	3

Abbreviations: ND, not detectable; t, trace.

<sup>a</sup>Fatty acid nomenclature: Prefix “i” indicates *iso*-branched fatty acid. The first number indicates carbon chain length. The number following the colon is the number of double bonds present with the double bond position from the methyl end of the molecule indicated by the number following the “ $\omega$ ”. Suffix “c” indicates the fatty acid is of the *cis* isomer.

<sup>b</sup>Trace fatty acids present at less than 0.5% of the total. Only major fatty acid components are shown. No data are available for *P. pulmonis*.

identification of the genus. The predominance in monounsaturated fatty acids is likely an adaptive factor for growth at low temperature since the low melting points of monounsaturated fatty acids helps maintain cytoplasmic membrane permeability at low temperature (Russell and Nichols, 2000). Other fatty acid components consistently detected include 18:0 (octadecanoic acid) and 3-OH 12:0 (3-hydroxy-dodecanoic acid). It should be noted that the presence of various minor components and the levels of components are significantly influenced by cultivation conditions and analysis methods used.

In *Psychrobacter* species, the major quinone is ubiquinone-8 (Q-8) and the major polyamines are spermidine and putrescine (Hamana and Takeuchi, 1998; Kämpfer et al., 2002). The polar lipids for the genus (phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol) are typical of Gram-negative bacteria. *Psychrobacter* species also contain unidentified aminolipids, glycolipids and aminophospholipids (Denner et al., 2001; Kämpfer et al. 2002). Wax esters are also a common feature of the genus (Bowman et al., 1996) and the family Moraxellaceae (Bryn et al., 1977) but rare in other bac-

teria. Analysis of this lipid class is aided by the use of iatroskan thin layer chromatography, which allows separation of different derivatized and underivatized lipid classes on a thin rod of silica before volatilization and direct analysis by standard flame ionization gas chromatography (Volkman and Nichols, 1991). The wax esters are usually C<sub>32</sub>–C<sub>38</sub> chains consisting of two fatty acids linked together by an ester bond. The production of wax esters may be tied to cold adaptation (Russell and Volkman, 1980), however the evidence for this is contradictory. The strictly mesophilic genus *Moraxella* is also rich in wax esters, which suggests wax esters may have another role in cell physiology. Levels and types of wax esters vary heavily between strains and species (Bowman et al., 1996).

## Genotypic and Genomic Characteristics

*Psychrobacter* species have a DNA G+C content of 41–51 mol% (Table 4), which is similar to the other genera of the family Moraxellaceae. For *Moraxella*, the range is 40–47 mol% and for *Acinetobacter*, 38–47 mol% (Bøvre, 1984). The values are much lower than that of halomonads and pseudomonads (55–65 mol%).

Except for some of its cold-active enzymes, *Psychrobacter* has been the subject of very little genetic analysis (see Applications section). However, a strain of *Psychrobacter* (strain 273-4; most closely related to *P. proteolyticus*) isolated from an approximately 30,000-year-old Siberian permafrost core (Vishnivetskaya et al., 2000) has now a draft genome available Oak Ridge National Lab Genomics Web site (<http://genome.ornl.gov/microbial/psyc/>). The genome is 2.6 Mb long with 2603 predicted open reading frames (ORFs). The genome contains five copies of the ribosomal RNA coding operon. The sequences and predicted homologous genes are available on the listed Web site and at the National Collection of Biotechnology Information Web site.

## Pathogenicity

Various *Psychrobacter* species have been found to occasionally cause infections in humans, animals and fish. The mechanisms of pathogenesis and epidemiology of pathogenic strains is essentially unknown. Transmission appears to be mainly in the form of environmental and nosocomial contact (e.g., breathing aerosols, wound infections, etc.). *Psychrobacter* is considered to be thus purely an opportunistic pathogen and

generally causes secondary albeit very serious infections. *Psychrobacter* has been found to cause rarely chronic peritonitis in humans (Daley et al., 1997) and these pathogenic isolates were designated “CDC group EO2” or “EO3” strains (Hudson et al., 1987; Moss et al., 1988). Various strains of *P. phenylpyruvicus* have been noted to cause infant meningitis and general bacteremia (Kikuchi et al., 1978; Bøvre, 1984; Lloyd-Puryear et al., 1991; Guttigoli and Zaman, 2000). *Psychrobacter* strains also cause ocular infections such as keratinoconjunctivitis (Gini, 1990), as well as endocarditis (Guttigoli and Zaman, 2000) and infective arthritis (Pérez et al., 2002). A *Psychrobacter* strain was implicated in a fatal infection of an acquired immunodeficiency syndrome (AIDS) patient (Lozano et al., 1994) and may cause lung infections in sheep (Vela et al., 2003). *Psychrobacter immobilis* strains can also be an opportunistic pathogen of farmed salmonid fish causing nonfatal secondary infections (fatal after 35 days) and a multitude of external and internal symptoms (Hisar et al., 2002). The antibiotic sensitivity of *Psychrobacter* strains is poorly known, but from the study of Romanenko et al. (2002), nonclinical strains were found to be resistant to most penicillin derivatives such as ampicillin and benzylpenicillin but sensitive to various aminoglycosides like kanamycin, neomycin and streptomycin.

## Growth and Isolation

*Psychrobacter* species as a whole can be grown on various organic rich media commonly used in the laboratory (Table 1). Best results are obtained when these media are supplemented with 0.5–1.0 M NaCl, as some strains require sodium for growth. Incubation temperatures vary with the species, but almost all grow rapidly at 20–25°C (Table 1). Most species grow very well on marine agar (formula below). Excellent growth for most species occurs on nutrient and trypticase soy agar (TSA), especially when these media are amended with an extra 25 g of NaCl per liter. Some clinical species such as *P. phenylpyruvicus* and *P. pulmonis* include some fastidious strains, which show poor or no growth on the media mentioned. Instead these can be grown readily on brain heart infusion (BHI), Columbia blood agar, or nutrient and TSA supplemented with 5% (v/v) horse serum; after incubation in a humid atmosphere at 30–37°C. CO<sub>2</sub> is not required. All media can be purchased as dehydrated powders or in prepared form from various commercial vendors (e.g., Difco, Oxoid, Gibco, etc.).

*Psychrobacter* can be isolated from source material (see Habitat and Ecology section; Table

1) by direct plating onto appropriate media, typically marine agar for marine samples (seawater, sea-ice, sediment etc.), nutrient agar or TSA (amended with 6% NaCl to exclude non-halotolerant species) for terrestrial samples, and BHI or blood/serum agar for clinical specimens. Incubation at lower temperatures for samples from more temperate climes will help strongly select for *Psychrobacter* strains. Increasing NaCl concentration in media to about 0.5–1.0 M or greater can also increase selectivity. For ancient glacial ice, permafrost and sea-ice samples, pre-enrichment in standard or dilute media (such as 1/10 strength nutrient agar) at low temperatures (–5 to +5°C) enhances the probability of obtaining isolates. This enhancement does not occur if samples are simply directly plated (Bowman et al., 1997b; Vishnivetskaya et al., 2000).

Largish, cream colored, smooth colonies with a buttery consistency appearing on plates are characteristics presumptive of *Psychrobacter* strains. This can be further affirmed by cells having a coccibacilli morphology, Gram-negative or variable staining, and ability to grow at 4°C and tolerate ~6% NaCl (w/v). Species identification is possible by phenotypic characterization and lipid analysis (Tables 2–5), but 16S rRNA sequencing may be needed to get a definitive identification in some cases.

#### Marine Agar

Bacteriological peptone	5 g
Yeast extract	2 g
Ferric pyrophosphate	10 mg
Sea salts	35 g
Agar (optional)	15 g
Distilled water	1000 ml

Alternatively, replace sea salts and distilled water with natural seawater. Adjust pH to 7.3–7.5. Autoclave 121°C, 15 min.

The dehydrated medium known as “Marine 2216 agar or broth” is available from Difco Laboratories Inc.

#### Artificial Seawater (ZoBell, 1946)

NH <sub>4</sub> NO <sub>3</sub>	2 mg
H <sub>3</sub> BO <sub>3</sub>	27 mg
CaCl <sub>2</sub>	1.14 g
FePO <sub>4</sub>	1 mg
MgCl <sub>2</sub>	5.14 g
KBr	0.1 g
KCl	0.7 g
NaHCO <sub>3</sub>	0.2 g
NaCl	24.3 g
NaF	3 mg
Na <sub>2</sub> SiO <sub>3</sub>	2 mg
Na <sub>2</sub> SO <sub>4</sub>	4.1 g
SrCl	26 mg
Distilled water	1000 ml

Sea salts can also be purchased from chemical companies such as Sigma-Aldrich or from aquarium suppliers (the latter source is far cheaper and has a similar level of quality).

## Preservation

*Psychrobacter* species form stable cultures and so can be maintained on agar plates or slants at normal growth temperature for 1–3 weeks or at 2–4°C for several months and potentially years. A temperature of 2°C is more ideal than 4–5°C, as fungal contamination is reduced and survival for cold adapted species is enhanced. Antifungal compounds, such as nystatin, should be added to media to prevent fungal contamination. Marine agar is an excellent media for agar storage for most species. Other media as noted in Table 1 and above should be used for species not able to grow well on marine agar. Low nutrient media or media containing large amounts of carbohydrates should never be used, as strains will rapidly lose viability. Heavy cell suspensions of *Psychrobacter* strains in broth mixed with 10–30% glycerol (v/v) can also be cryopreserved for several years by first pre-chilling on ice and then direct transfer of the suspensions to a –70°C or colder freezer. The suspensions can also be frozen directly in liquid nitrogen at –196°C. The cultures should be replaced on a regular basis (once every few years) if possible. Multiple vials of cryopreserved cells should be stored in one freezer in addition to a back-up set stored in a separate freezer. Lyophilization can also be employed using a variety of desiccants including skim milk, horse serum, or fetal bovine serum.

## Habitats and Ecology

The genus *Psychrobacter* is found in a wide range of habitats (Table 1; Fig. 1). Most of the studied species have been isolated from cold, saline environments. For example, sea-ice and icy coastal seawater are excellent habitats for many *Psychrobacter* spp. (Bowman et al., 1997a; Bowman et al., 1997b; Bozal et al., 2003; Brinkmeyer et al., 2003; Yumoto et al., 2003). *Psychrobacter* strains have also been isolated from ancient glacial and ice sheet cores (Christner et al., 2003), supercooled water brine lenses in permafrost (cryopegs) derived from ancient marine layers of the Arctic Ocean (Vishnivetskaya et al., 2000; Gilichinsky et al., 2003), and accreted ice at the base of deep ice cores above seawater (Bowman et al., 1997a). In these apparently totally frozen locations, liquid can still occur at temperatures down to –10 to –15°C owing to the high salt (as high as 170–300 practical salinity units [psu]) concentrated by freezing. Carbon sources have been shown to be metabolized at –15°C in such places, and thus these exceptionally extreme environments have been suggested as useful models for astrobiology (Gilichinsky et al., 2003).



*Psychrobacter* species are also readily isolated from conventional marine environments including seawater (Venkateswaran et al., 1991; Kisand et al., 2002; Romanenko et al., 2002), marine sediment, and salt marshes where they may play a role in the breakdown of dimethylsulfoniopropionate (DMSP), a major osmolyte of algae (Ansedè et al., 2001). DMSP is broken down to dimethylsulfide, which enters the atmosphere where theoretically it influences weather by helping to initiate cloud formation. *Psychrobacter* species, such as *P. pacifiensis* also occur in sea depths of up to 6000 m (Maruyama et al., 1997; Maruyama et al., 2000) and seafloor sediments (Inagaki et al., 2003); however, as far as is known *Psychrobacter* species do not possess adaptations to high pressure. *Psychrobacter* strains have also been shown to occur on unusual calcium carbonate column formations (called “ikaite tufa” in Greenland fjords; Stougaard et al., 2002). These strains could be seawater derived.

*Psychrobacter* spp. are also an important component of the food microbiota as mentioned previously in the Taxonomy section. This high prevalence is connected obviously to the combined psychrotolerance and osmotolerance of *Psychrobacter* species. *Psychrobacter* species are especially common on salted fish, chilled fish flesh, some shell fish, fermented seafood, chilled meat products of all varieties, and even cheese and raw milk (e.g., Gennari et al., 1992, Gennari et al., 1999; Prieto et al., 1992; Garcia-Armesto et al., 1993; Pin and Baranyi, 1998; Gonzalez et al., 2000; Pacova et al. 2001; Sakala et al., 2002; Vasquez et al. 2002; Bagge-Ravn et al., 2003; Bjorkevoll et al., 2003; Yoon et al., 2003). Though *Psychrobacter* spp. can spoil food, they are considered relatively minor spoilers compared to other bacterial species. In fish, *Psychrobacter* spoilage results in a musty off-odor, usually after the fish has been stored in the cold for 7–10 days. The offensive odors are not from compounds like trimethylamine *N*-oxide or H<sub>2</sub>S, typically produced by major spoilers like *Shewanella* species. Also they do not produce histamines, which causes allergy problems for some consumers. Unlike most spoilers, *Psychrobacter* will tolerate salting and can survive storage in 25% NaCl for prolonged periods (Bjorkevoll et al., 2003). *Psychrobacter* appears to be mostly part of the normal surface microbiota of fish skin or the skin of various other animals. It can be found in the tissues of marine animals and sponges and on seaweeds and algae (e.g., Denner et al., 2001; Pukall et al., 2001; Romanenko et al., 2002). *Psychrobacter* also seem to be common in uric acid-rich soil derived from bird guano (e.g., Antarctic ornithogenic soils; Bowman et al., 1996) and in fecal bioaerosols (Kämpfer et al., 2002). *Psy-*

*chrobacter* species are otherwise absent from most soil environments unless they are exposed to continual low temperature and intermittent freezing (e.g., Wery et al., 2003).

Little is known specifically about the ecology of genus *Psychrobacter*. It is likely the genus has a role primarily as a commensal, degrading various dissolved organic carbon compounds other than sugars. The genus has evolved to tolerate low temperature and has a highly developed osmotolerance which allows it to be highly competitive and prevalent in many cold ecosystems.

## Applications

Since *Psychrobacter* strains are thoroughly cold adapted, most biotechnological interest has been in novel cold-active proteins and enzymes which have broad applicability in industry and been extensively reviewed (e.g., Bull et al., 2000; Rothschild and Mancinelli, 2001). Important focus areas for cold-active enzymes include janitorial processes, modification of heat labile substances (such as various foods), and in energy conservation. Many of the proteins studied are quite novel. For example, *Psychrobacter* strains have been found to be a source for new protein families including cold-active glutamate dehydrogenases (Camardella et al., 2002) and anti-freeze proteins (Gilbert et al., 2004). Other cold-active enzyme studies have involved metalloprotease (Denner et al., 2001), lipase (Arpigny et al., 1997), and β-lactamase (Feller et al., 1997). *Psychrobacter* strains have also been shown to possess cold-active rolling circle recombination replication proteins (Duilio et al., 2001), including novel restriction endonuclease methyltransferases (Rina et al., 1997), which may have application in genetic modification technology.

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## The Genus *Leucothrix*

THOMAS D. BROCK

*Leucothrix*, a large-diameter, morphologically distinct, marine gliding bacterium, has been known in natural material since the time of Oersted in 1844, but its modern history began with the remarkable study Harold and Stanier published in 1955. In the introduction to their paper, Harold and Stanier state: “*Leucothrix* may be characterized succinctly as a chemoheterotrophic counterpart of the colorless sulfur-oxidizing organism *Thiothrix*. It has been observed on a few occasions . . . in the century since its original description, but the existing accounts of its morphology and development, based entirely on the examination of crude cultures, are either incomplete or inaccurate. Thanks to the ease with which it can be grown in pure culture, we have been able to determine its complete cycle of development, which includes a unique and hitherto undescribed process of gonidial aggregation to form many-celled rosettes.”

*Leucothrix* is fascinating not only because of its morphological distinctiveness, but also because of its large size and the ease with which it can be recognized in natural material. The organism seems to be entirely marine and is widespread as an epiphyte of marine algae (Fig. 1) (Brock, 1966). It also causes an extensive infestation of benthic crustacea and fish eggs (Johnson et al., 1971), and has become a problem in the field of aquaculture, especially in the artificial cultivation of lobsters. *Leucothrix* appears to be related to the cyanobacteria (Pringsheim, 1957; Raj, 1977), as determined by morphological similarities. However, no detailed studies of molecular relationships between *Leucothrix* and any cyanobacteria have been carried out, although the DNA base composition of *Leucothrix mucor* isolates, 47–49 mol% GC (Brock and Mandel, 1966), is similar to that of a number of filamentous cyanobacteria (Edelman et al., 1967), which cluster in the range 42–51 mol% GC. This similarity is made even more striking when it is compared with the wide variation in DNA base compositions of the unicellular

cyanobacteria (Edelman et al., 1967; Stanier et al., 1971) and with the wide variation found in the narrow-diameter gliding bacteria (Edelman et al., 1967).

### Characteristics of *Leucothrix*

A simplified life cycle of *Leucothrix mucor* is given in Fig. 2. *Leucothrix* filaments are usually 2–3  $\mu\text{m}$  in diameter and may reach lengths of 0.1–0.5 cm. The filaments have clearly visible cross-walls, and cell division is not restricted to either end but occurs throughout the length of the filament, as shown by autoradiography with tritiated thymidine (Brock, 1967). The free filaments never glide (thus distinguishing them from many other filamentous gliding bacteria, such as *Beggiatoa* and *Vitreoscilla*), although they occasionally wave back and forth in a jerky fashion. Under environmental conditions unfavorable to rapid growth, such as low temperature or low nutrient concentration, individual cells of the filaments become round and form ovoid structures called “gonidia,” which are released individually, often from the tips of the filaments (Fig. 3). The gonidia are able to glide in a jerky manner when they come into contact with a solid surface. They settle down on solid surfaces, synthesize a hold-fast, and form new filaments through growth and successive cell divisions. In nature, the gonidia are presumably elements of dispersal and enable the organism to spread to other areas. “Gonidia,” first used by Winogradsky (1888) for *Thiothrix*, is an unfortunate term because it implies some sort of unique structure. Actually, the gonidium of *Leucothrix* and *Thiothrix* is functionally and structurally equivalent to the hormogonium of the cyanobacteria, which is a motile structure of dispersal formed by the rounding up of a vegetative cell or group of cells in a filament.

Both Winogradsky (1888) for *Thiothrix* and Harold and Stanier (1955) for *Leucothrix* concluded that an apical-basal differentiation of filaments existed and that gonidia were formed primarily at the tips of filaments. Actually, such an apical-basal differentiation does not exist, and filaments do not taper. Careful examination of

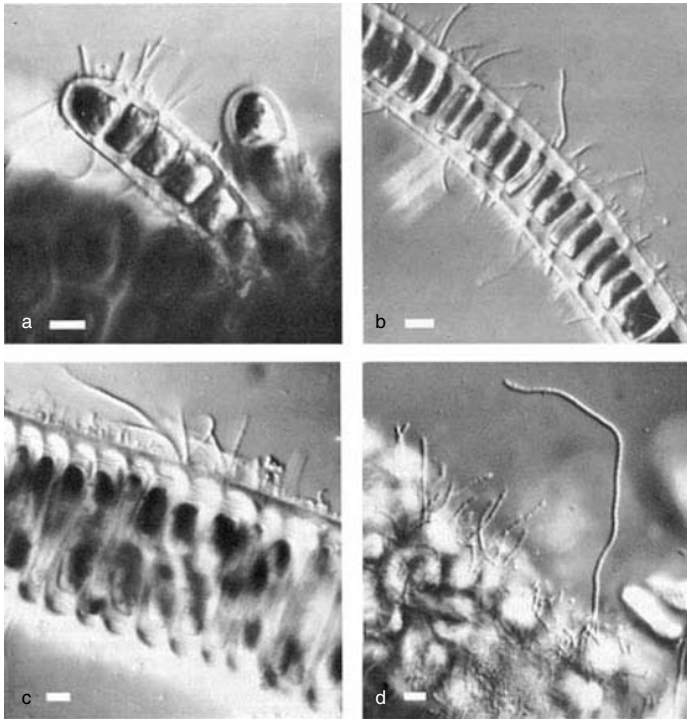


Fig. 1. Filaments of *Leucothrix mucor* attached to fronds of the seaweed *Bangia fuscopurpurea* of various ages. (a) Very young, (b) young, (c) mature, (d) old. Nomarski interference contrast photomicrograph. Bars = 10 µm. (From Bland and Brock, 1973.)

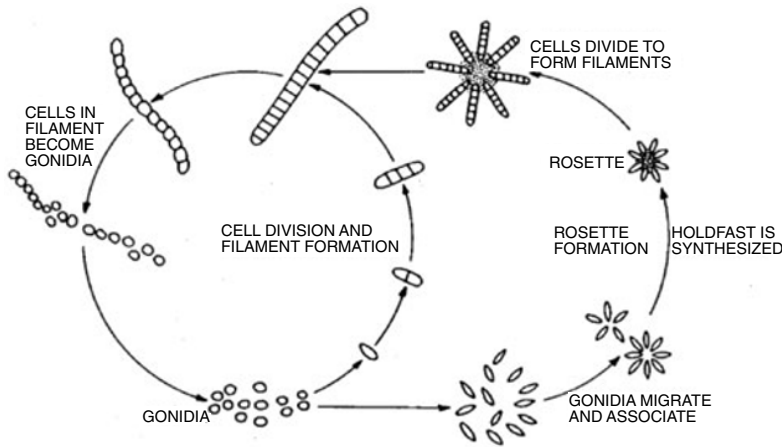


Fig. 2. Life cycle of *Leucothrix mucor*.

even long filaments has never revealed narrower cell diameters at the ends than in the middle. Also, although gonidia may form primarily at the filament tips, this is by no means always the case. I have frequently seen whole filaments convert to a series of gonidia, with every cell in the filament rounding up.

Gonidia do not have a holdfast when first formed, but make it only in response to the proper environmental conditions. If there are high concentrations of gonidia, individual cells may aggregate, probably because of reciprocal attraction (chemotaxis?). They then synthesize a holdfast and the gonidia become adherent to each other by the holdfast and form a small rosette. A new filament grows out from each

gonidium, eventually resulting in a large and striking structure (Fig. 4). Rosette formation is found in both *Leucothrix* and *Thiothrix* and is an important means of distinguishing these organisms from many other filamentous bacteria.

Another interesting characteristic of *Leucothrix* is the ability of filaments to grow in such a way that knots are formed (Fig. 5) (Brock, 1964). Knots occur mainly when the organism is growing in rich liquid culture media, where filamentous growth is rapid. Knot formation is frequent enough in *Leucothrix* cultures to be used as a taxonomic characteristic. Since individual filaments do not flex or glide, a preformed filament cannot form a knot. Actually, knots seem to be

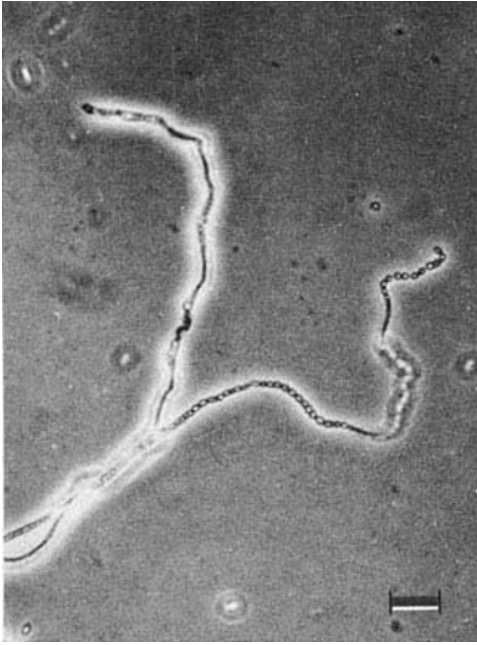


Fig. 3. Release of gonidia from filaments of *Leucothrix mucor*. Phase-contrast photomicrograph. Bar = 10  $\mu\text{m}$ .

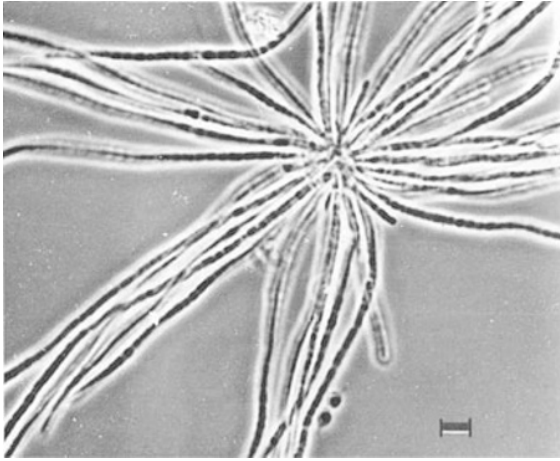
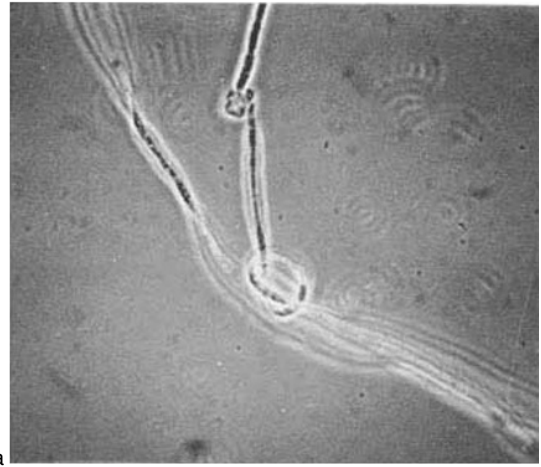
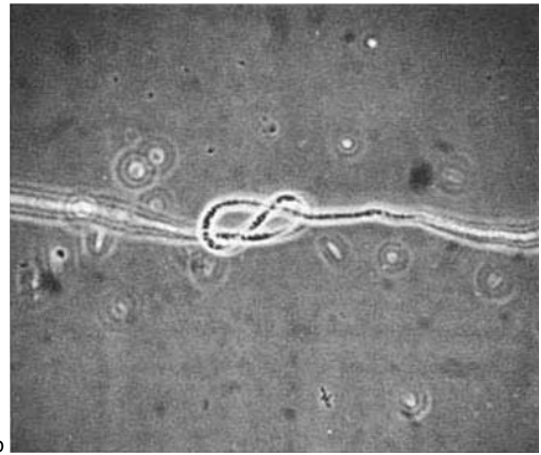


Fig. 4. Rosette composed of several *Leucothrix* filaments. Phase-contrast photomicrograph. Bar = 10  $\mu\text{m}$ .

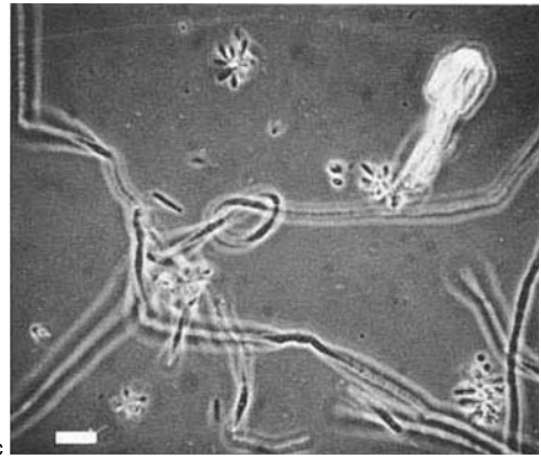
formed as part of the growth process, probably because growth occurs faster on one side of the filament than on the other, causing the filament to form a loop through which the tip of the filament can pass. Once the knot is formed it cannot be untied; rather, the cells in the region of the knot eventually fuse and form a bulb that is later released from the rest of the filament. As a result, the long filament is separated into two shorter filaments. Knots are also seen occasionally in *Thiothrix* filaments in sulfur springs, where large accumulations of these organisms appear, and in *Leucothrix* populations in nature (T. D. Brock,



a



b



c

Fig. 5. Knots formed by filaments of *Leucothrix mucor*. Phase-contrast photomicrograph. Bar = 5  $\mu\text{m}$ . (From Brock, 1964.)

unpublished observations). All of the structural features of the *Leucothrix* life cycle can be observed not only in culture but also in natural material, especially when Nomarski optics are used to examine the surfaces of algal fronds (J. A. Bland, personal communication).

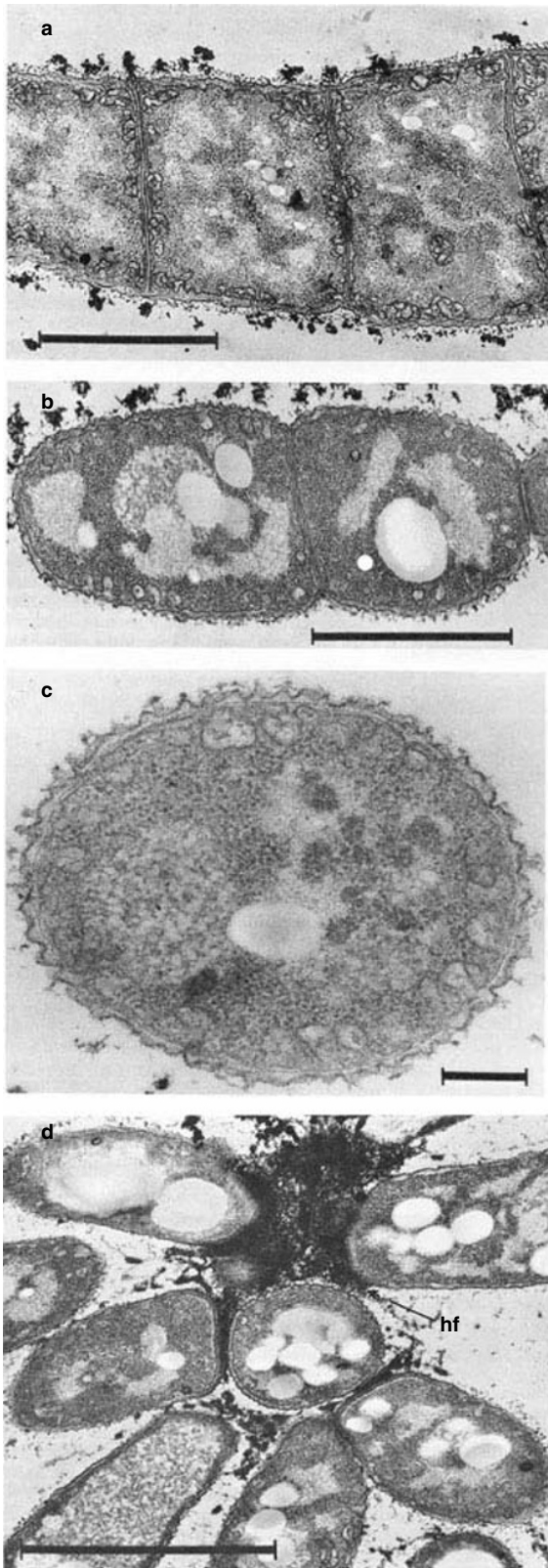


Fig. 6. Electron micrographs of thin sections of *Leucothrix mucor*. (a) Portion of a multicellular filament. (b) Filament in early stage of gonidial formation, showing rounding up of the cells. Note that there is no change in the ultrastructure of the wall. (c) A single gonidium. The gonidia retain all of the envelope structures observed in filaments. (d) Thin section through a rosette. The holdfast material (hf) is an electron-dense material surrounding the cells. (a, b, d) Bars = 1.0  $\mu\text{m}$ ; (c) bar = 0.2  $\mu\text{m}$ . (From Brock and Conti, 1969.)



An electron microscopic study of *Leucothrix* was carried out by Brock and Conti (1969), and some of the structural features of the organism are seen in Fig. 6.

### Taxonomy of *Leucothrix*

The question of the physiological or taxonomic relationship between *Leucothrix* and *Thiothrix* has not been resolved. A *Thiothrix* filament or rosette that has lost its sulfur granules cannot be distinguished from *Leucothrix*. Harold and Stanier (1955) placed pure cultures of *Leucothrix* in sulfide-containing sea water and examined them at intervals for the presence of intracellular sulfur granules. No evidence of sulfur accumulation was obtained; this was also my experience with one pure culture. However, Kjell Eimhjellen of the Technical University in Trondheim, while working in C. B. van Niel's laboratory at Pacific Grove, California, was successful in attempts to show oxidation of hydrogen sulfide by *Leucothrix*. In his words (personal communication, 26 March 1970):

*Leucothrix mucor* (the strains I isolated all corresponded very well to the description of Harold and Stanier) pre-grown on the usual media, can be made to oxidize sulphide when exposed to an atmosphere of hydrogen sulphide and oxygen, very much in the manner of *Sphaerotilus* observed by Skerman. Based on comparison of the pictures published by Skerman, *Leucothrix*, however, seemed to have a greater ability to oxidize sulphide. The filaments of *Leucothrix* often had a very heavy accumulation of internal S-globules, making the filaments resemble the very best rosettes of *Thiothrix* you can find in nature. After extensive washing of such cells to remove chemically produced sulphur, resuspension in low-sulphate media and incubation by gentle shaking, the internal S-globules disappeared and copious amounts of sulphate could be isolated from the supernatant. The sulphide oxidation was never followed manometrically, but I did a great many measurements of the ability of *L. mucor* to oxidize thiosulphate. Every time the oxidation would proceed quantitatively to tetrathionate and in a fair number of experiments the oxidation went further to a complete oxidation to sulphate. I could never clear up the reasons for this inconsistency. In separate experiments, the presence or absence of carbon dioxide seemed to make no difference on the total oxygen uptake, indicating no use of thiosulphate as electron donor for  $\text{CO}_2$  fixation. . . . My conclusion is that the strain of *L. mucor* I used had the enzymatic capability to oxidize



sulphide, sulfur and thiosulphate, all compounds being oxidized to sulphate, but no evidence for any energy link has been found.

It seems likely from a comparison of the results of Eimhjellen with those of Harold and Stanier (1955) that strain differences exist and that some strains of *Leucothrix* are able to deposit elemental sulfur. Further work on this problem would be of considerable interest.

Only a single species of *Leucothrix* is currently recognized, *Leucothrix mucor* (Brock, 1974). Pringsheim (1957) described *L. cohaerens* as a second species, based on cell diameter and filament length, but there is considerable variation in these properties even in a single pure culture, and since Pringsheim's isolates are no longer available, it seems preferable to maintain only a single species. Kelly and Brock (1969b) showed by physiological and molecular techniques that a wide variety of *Leucothrix* isolates, obtained from coastal areas around the world, were very similar. Of 35 strains characterized, all had a pH optimum of 7.6 and a salinity optimum of 30 parts per thousand. (The GC content of the DNA varied only over the narrow range of 48–51 mol% GC. Temperature optima of all strains were in the range 25–30°C, with a maximum of 33°C. Most strains showed a minimum temperature for growth of 0–2°C, although one strain, isolated from a tropical area, had a minimum temperature for growth of 13°C and hence was more stenothermal. Growth factor requirements were determined for all 35 strains. Most had no growth factor requirements and were able to grow heterotrophically on a simple glutamate culture medium, but 2 strains required thiamine, 1 strain (the warm water isolate) required vitamin B<sub>12</sub>, and 3 strains showed a partial aspartate requirement. All the strains possessed the array of morphological characteristics typical of the species: filaments, gonidia, rosettes, knots, and bulbs. Brock described a neotype strain of *Leucothrix mucor* and listed its characteristics as follows.

### Morphology

Filaments are of variable length, often much greater than 100 µm, with a diameter of 2–3 µm. Sulfur granules are not formed. True knots are usually formed by pure cultures when growing in organically rich media. Filaments are colorless, unbranched, and nonmotile (although occasionally waving back and forth) and lack a sheath, although cells in regions of a filament may become emptied of their contents and give the appearance of a sheath. Filaments often grow intertwined or in dense tangles. Swollen cells often form at random along filaments. Larger

structures (bulbs) usually form in knotty cultures, probably as a result of fusion of cells in the region of the knots. Filaments are attached to solid substrates by means of an inconspicuous holdfast that can be seen by staining with primulin and viewing with blue light in a fluorescent microscope; the holdfast fluoresces red. Individual filament cells round up and form ovoid to spherical gonidia that acquire a jerky gliding motility when released. Gonidia frequently aggregate in cultures, probably chemotactically, to form rosettes.

### Nutrition

Most strains do not require growth factors. They grow on glutamate as sole source of carbon, nitrogen, and energy. They also use sugars, organic acids, and other amino acids as carbon and energy sources and NH<sub>4</sub><sup>+</sup> as nitrogen source. *Leucothrix* requires Na<sup>+</sup> for growth (optimum 1.5% NaCl, minimum 0.3%, maximum 7%).

### Relation to Temperature

Optimum 25–28°C; maximum 32–35°C. *Leucothrix* grows at 0°C to form visible colonies within 1–2 weeks.

### Relation to O<sub>2</sub>

Obligately aerobic.

### Habitats

As noted in the Introduction, *Leucothrix* appears to be strictly a marine organism. It is widespread as an epiphyte on marine algae and also occurs as an infestation of benthic crustacea (Johnson et al., 1971). On seaweeds, it appears most commonly in temperate waters (Kelly and Brock, 1969a), where densities are often quite high. In a detailed study at Friday Harbor, Washington, Bland and Brock (1973) made quantitative microscopic determinations of *Leucothrix* densities on a number of seaweeds. The organism was found on a variety of red, green, and brown algae, but was much more abundant on intertidal than on subtidal algae. The red alga *Bangia fuscopurpurea*, a filamentous species living in the high intertidal region, was unusually heavily colonized, with *Leucothrix* populations 10–30 times larger than those on other algal species in the same locations. Because of its high intertidal location, *B. fuscopurpurea* is exposed to air for fairly long periods during each tidal cycle. *B. fuscopurpurea* mats, dried during low tide, showed greater *Leucothrix* populations on the underside of the mats than on the top, presumably because

the underside of the mats retained moisture longer. Examination of *B. fuscopurpurea* filaments of various ages showed that there was a continuous increase in the *Leucothrix* density with algal age, a finding that suggested growth on the alga itself. Using axenic two-membered cultures in an autotrophic medium with several red algae (including *B. fuscopurpurea*) and *L. mucor*, Brock (1966) showed that *Leucothrix* could grow on nutrients produced or liberated from the alga. Bland and Brock (1973) used artificial substrates and a variety of ecological studies to demonstrate that in nature *Leucothrix* obtained most of its nutrients from the alga and not from the sea water. Details of these ecological experiments and a review of other literature on the ecology of *Leucothrix* should be sought in the original paper (Bland and Brock, 1973).

Brock (1966) thought that *Leucothrix* was more abundant on seaweeds in temperate than in tropical waters (see also Kelly and Brock, 1969c), but Johnson et al. (1971) found *Leucothrix* to be abundant on tropical algae in the Pacific. Brock (1966) also concluded that *Leucothrix* was more abundant on algae in habitats of high aeration, such as open, exposed coasts and areas with heavy tidal currents. Since *Leucothrix* is an obligate aerobe, the preference for highly aerated habitats is not surprising. The finding that *Leucothrix* prefers algae in the intertidal rather than the subtidal zone is in keeping with this hypothesis, although there are, of course, factors other than aeration that differ in these two kinds of habitats. A survey of marine habitats showed that *Leucothrix* was worldwide in distribution (Kelly and Brock, 1969b), including the southern hemisphere (Brock, unpublished observations in New Zealand).

An authentic freshwater strain of *Leucothrix* has never been isolated, although *Thiothrix* occurs in both fresh and marine waters. Microscopic surveys of freshwater algae, even freshwater red algae, have never shown any indication of the presence of *Leucothrix* (Brock, unpublished observations), although Eikelboom (1975) found a number of *Leucothrix*-like organisms in activated sludge (see also van Veen, 1973; Sladka and Ottova, 1973). However, because there are various other filamentous septate bacteria, positive confirmation of freshwater *Leucothrix* will require isolation in pure culture and demonstration of the various stages of the life cycle, especially gonidia and rosettes.

## Isolation

Harold and Stanier (1955) first isolated *Leucothrix* from crude enrichment cultures of rotting

seaweeds, but such cultures are generally grossly contaminated and purification of *Leucothrix* is often difficult. A more effective way of isolation (true for many other gliding filamentous bacteria as well) is to avoid enrichments completely and carry out a direct, single-colony isolation from natural material. This operation is made especially easy with *Leucothrix* when it is growing on relatively clean seaweeds, because on such samples, motile unicellular contaminants (the organisms presenting the greatest difficulty in isolation) are relatively small in number.

Any marine-type seawater base can be used in the formation of a culture medium; natural sea water (filter-sterilized) itself can be used, but pH control and avoidance of metal precipitation are easier in a Provasoli-type culture medium (Provasoli, 1963). The following salts formulation (Brock, 1966) proved quite effective:

NaCl	11.75 g
MgCl <sub>2</sub> · 6H <sub>2</sub> O	5.35 g
Na <sub>2</sub> SO <sub>4</sub>	2.0 g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.75 g
KCl	0.35 g
Tris(hydroxymethyl) aminomethane	0.5 g
Na <sub>2</sub> HPO <sub>4</sub>	0.05 g
Deionized water	1 liter
Adjust to pH 7.6.	

The low phosphate concentration of this salts base is critical, as media of normal phosphate levels are inhibitory. Most strains have no vitamin requirements and use glutamate as the sole source of carbon, nitrogen, and energy, so that this salts medium with 0.1% monosodium glutamate (MSG) and 2% agar (to inhibit swarming organisms) will permit the isolation of *Leucothrix* from most materials. Other useful media include 0.1% MSG plus a vitamin mixture, 0.1% MSG plus 0.01% yeast extract, and 0.1% tryptone plus 0.1% yeast extract. In the initial isolation step, it is best to keep the organic concentration of the medium low to avoid problems with overgrowth by unicellular bacteria.

For isolation, single algal filaments are streaked directly (or after washing in sterile salts) onto agar plates, which are incubated at 20–25°C overnight. Within 12–18 h after streaking, the plates are examined under 125× magnification (10× eyepiece with a long working distance, phase-contrast objective is best) for the presence of characteristic *L. mucor* colonies, which have a coiled rope or thumbprint morphology. These colonies are picked by touching them with a sterile insect pin and transferring them to fresh agar plates of the same composition. It is important that colonies be identified early after inoculation, before the plates are overgrown. Although a dissecting microscope can also be used to locate *Leucothrix* colonies

(Harold and Stanier, 1955), I have found that a 10× microscope objective (12.5× eyepiece) is better for locating colonies when they are very small (before they are overgrown). By using these procedures, it has been possible not only to isolate *Leucothrix* from seaweed from a wide variety of habitats, but also, with very clean material, to obtain growth of *Leucothrix* colonies directly from filaments still attached to seaweed fronds. In this way, the precise habitat from which the isolate was obtained is known; such information is of considerable value in studies on the molecular evolution of *Leucothrix* (Kelly and Brock, 1969b).

During transfer of colonies to liquid culture, it has often been observed that only very slight growth, if any, occurred after several days when an inoculum was transferred from agar to 100-ml liquid cultures; but when the inoculum was placed in a small volume of medium, such as 1–2 ml, heavy growth occurred overnight. Such small cultures could be easily used as the inoculum for large flasks and permitted the buildup of large-volume cultures. In liquid medium, growth is best when the flasks are shaken gently, such as on a wrist-action shaker or slowly on a rotary shaker. With the latter kind of shaker, growth rate is increased if the flasks contain small internal baffles, made by pushing in the sides of the flasks during heating with an oxygen flame.

For growing high-density cultures, a medium containing 1% MSG, 0.2% sodium lactate, and 0.01% yeast extract has proved quite suitable, the yeast extract providing growth factors needed by some strains. The sodium lactate was found to substantially increase the yield of most cultures.

## Identification

*Leucothrix* is identified simply on the basis of morphological examination with the light microscope. The filaments are of large diameter (greater than 2 μm; average, 2–3 μm), and each filament is composed of short cylindrical or ovoid cells, with cross-walls clearly visible. The filaments are colorless, unbranched, and of variable length, often very long. The filaments do not taper, but there may be variation in diameter along the length of the filaments. The filaments do not glide, although they may wave sporadically from side to side. Rosette formation is a key diagnostic characteristic. Without observation of the presence of rosettes, it is not possible to easily identify an isolate as *Leucothrix*. Conversion of cells in filaments into gonidia may result in the formation of chains of spherical cells, or gonidia may form only at the ends. The gonidia exhibit

gliding motility, although the rate of motility is generally so slow that gliding itself is not a good diagnostic characteristic. To study gonidia formation, gliding, and aggregation, slide cultures are preferable; chambers that permit examination with 10–25× objectives should be used. Growth in such chambers is better if a considerable air space is left along one side, since *Leucothrix* is an obligate aerobe.

Filaments in culture often form true knots, and the presence of knots in a culture may be considered indicative of *Leucothrix* even without the formation of rosettes. However, the density of knots is never high, and a number of microscopic fields must be searched to ascertain if knots are present. Knot formation is most frequent when growth occurs to a high cell density in a relatively rich culture medium.

All strains that have been isolated are marine and require NaCl for growth; but, since freshwater strains could theoretically exist, NaCl requirement should not be considered a diagnostic characteristic. The GC content of the more than 30 strains examined ranged from 46 to 51 mol%, a relatively narrow range. Only one species is recognized (Brock, 1974), *Leucothrix mucor*.

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## The Genus *Lysobacter*

HANS REICHENBACH

The species classified in the genus *Lysobacter* are Gram-negative rods that move by gliding. The cells are slender and cylindrical, with rounded ends (Figs. 1 and 2). They typically measure 0.4–0.6 × 2–5 μm, but in the population there are also always long to very long (up to 70 μm) cells and filaments. The cell shape and the occurrence of long cells are both very characteristic for the genus. *Lysobacter* cells resemble the vegetative cells of certain myxobacteria, specifically of the genera *Polyangium* and *Sorangium*, with which the lysobacters were confused for many years. They also share with the myxobacteria a high GC content of their DNA of 65 to 70 mol%. Due to the gliding movements of the cells, the colonies of *Lysobacter* are spreading or swarming on solid media and may become very large and extremely thin (Figs. 3 and 4). Sometimes the organisms produce copious amounts of slime, and the colonies then become thick and deliquescent, but colonies with a wrinkled and dry surface also occur. *Lysobacter* colonies may be white or cream-colored but often they are greenish-yellow, purplish-red, or brown, although their color is often rather pale. Some strains produce an unpleasant odor reminiscent of certain pseudomonads or of pyridine. In agitated liquid cultures, the lysobacters grow as homogeneous cell suspensions, but, as with all gliding bacteria, the suspended cells are unable to translocate. The *Lysobacter* species live in soil, decaying organic matter, and fresh water, sometimes in large populations. Many strains are of considerable ecological and biotechnological interest as producers of exoenzymes and of antibiotics.

The genus *Lysobacter* was defined by Christensen and Cook (1978) who also described the presently recognized species and created a new family, Lysobacteraceae, and a new order, Lysobacterales. The organisms thus classified had already been known, however, for a long time under various names, such as *Cytophaga*, *Sorangium*, and *Myxobacter* (the latter an obsolete myxobacterial genus), which were usually pre-

sented with some doubts of the investigators concerning the classification of their strains. The first lysobacter in the scientific literature may have been *Flexibacter albuminosus* (Soriano, 1945, 1947), which had the cell size and shape of a lysobacter and formed thick dirty-white colonies and a diffusible dark pigment. But the description is not accurate enough and the strains are no longer available so that the question cannot be decided. The first unequivocal *Lysobacter* strain was a chitinolytic strain first tentatively identified as *Cytophaga johnsonae* Veldkamp, (1955). It is deposited at the National Collection of Industrial Bacteria (NCIB no. 8501) and was originally listed as a *Polyangium* species. The strain has a GC content of 71 mol% ( $T_m$ ) and was noted as an unusual case of a cytophaga with a high GC content (Mitchell et al., 1969). Other early strains that later turned out to be lysobacters are: 1) “myxobacter” or “*Sorangium*” strain 495, which was studied because it attacks nematodes (Katznelson et al., 1964) and various bacteria (Gillespie and Cook, 1965) and contains very interesting proteases (for details, see “Practical Aspects,” this chapter); the strain also produces two peptide antibiotics, the myxosidins (Clapin and Whitaker, 1976, 1978); 2) “Myxobacter” AL-1, which became of interest because it digested cells and cell walls of *Arthrobacter crystallopoietes* (Ensign and Wolfe, 1965) and which was later found to excrete two unusual proteases; 3) “*Sorangium*” 3C, producer of the wide-spectrum phenazine antibiotic myxin (Peterson et al., 1966); 4) “*Cytophaga*” L1, (NCIB 9497) for which a patent was filed for a number of unusual enzymes of practical interest, e.g., keratinase, laminarinase, and chitinase (Brit. Pat. 1,048,887, 23 November 1966), and 5) “*Cytophaga johnsonae*” (ATCC 21123), originally isolated because of its lytic enzymes at Kyowa Hakko in Japan (Jap. Pat. 06624, 1969), and from which the new quinoline antibiotic G1499–2 was obtained (Evans et al., 1978). In addition a number of lysobacters, usually labeled “myxobacters,” were isolated because they attacked cyanobacteria and green algae and multiplied spectacularly

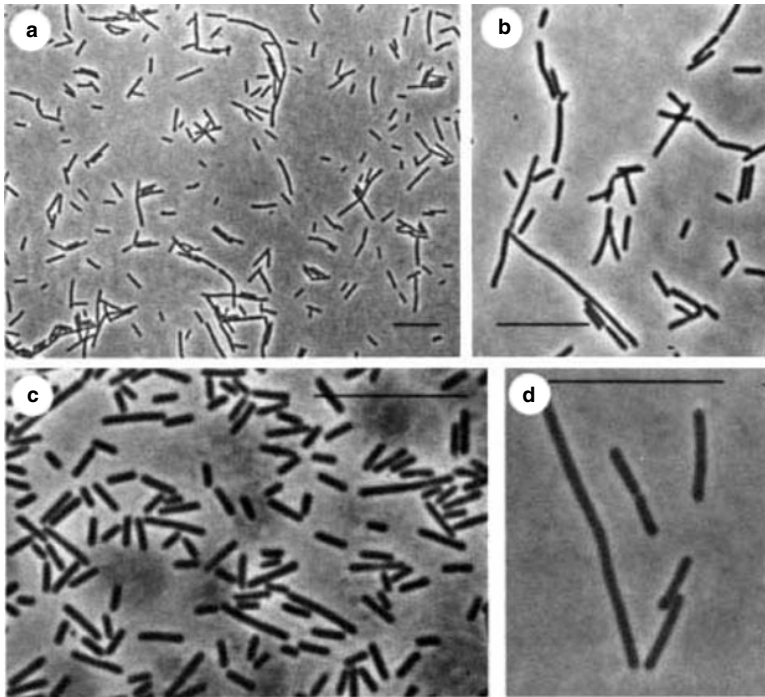


Fig. 1. Phase contrast photomicrographs of *Lysobacter*. (a), (b), and (d) *L. antibioticus* strain UASM L17 (= ATCC 29480) grown on CY agar (0.3% Casitone, 0.1% yeast extract) for 3 days at 20°C. Typical is a fairly regular cylindrical cell shape and a substantial variation in cell length. (c) *L. enzymogenes* type strain UASM 495 (= ATCC 29487), grown on VY/2 (yeast) agar for 13 days at 30°C. (a) Bar = 20 µm. (b), (c), and (d) Bars = 10 µm.

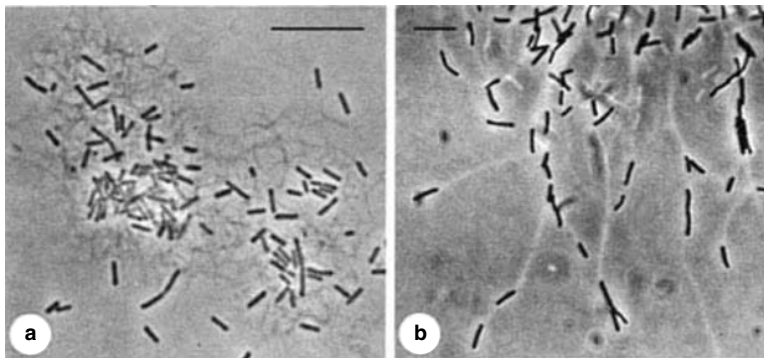


Fig. 2. Phase contrast photomicrographs of slime structures produced by *Lysobacter*. (a) *L. antibioticus* strain UASM L17 grown on CYG2 agar (CY agar plus 0.3% glucose) for 3 days at 20°C. For photography, the cells were transferred to a thin film of water agar. A network of slime threads is clearly visible. Bar = 10 µm. (b) The cyanobacteriolytic lysobacter of Shilo (1970). Edge of a colony on Shilo's (2% Casitone) agar in a chamber culture. Slime trails have been laid by the cells during their gliding movements. Bar = 10 µm.

during algal blooms. Thus, “myxobacter” FP-1 specialized on cyanobacteria (Shilo, 1967, Shilo, 1970); “*Cytophaga*” N-5, later renamed “myxobacter” 44, lysed cyanobacteria and green algae (Stewart and Brown, 1969); “myxobacters” 45 and 46, which with “myxobacter” 44, “*Sorangium*” 3C, and “myxobacter” AL-1, have an uncommonly high GC content of around 70 mol% (Stewart and Brown, 1971); and the cyanobacterium-lysing bacteria with a high GC content isolated from British waters, e.g., strains CP-1, -2, -3, and -4 (Daft and Stewart, 1971; Daft et al., 1975).

The phylogenetic position of the genus *Lysobacter* remained obscure until recently. To the

early investigators, gliding motility suggested some relationship with other, existing groups of unicellular gliding prokaryotes, specifically the myxobacteria and the cytophagas (Reichenbach, 1981). This is reflected by the names given to the strains isolated at that time. But as was already correctly anticipated in the taxonomic description of the new organisms, these bacteria form a group of their own (Christensen and Cook, 1978). Later, 16S RNA studies demonstrated that *Lysobacter* is relatively closely related with the xanthomonads and belongs to the gamma-3 branch of the purple bacteria (Woese et al., 1985) known today as the class Proteobacteria (Stackebrandt et al., 1988).

Fig. 3. The *Lysobacter* colony. Bars = 1 mm. (a), (b), and (c) *L. antibioticus* strain UASM L17 grown on different media for 7 days at 20°C: (a), on CA2 agar (0.075% KNO<sub>3</sub>, 1% glucose); (b), on MYX agar (0.5% Na glutamate, 0.1% yeast extract, 0.2% glucose); and (c), on CY agar. (d) and (e) the chitinoclastic strain (NCIB 8501) of Veldkamp (1955): (d) grown on CA2 agar; (e) grown on CY agar; both 7 days old (20°C). (f) *L. enzymogenes* strain UASM 495 grown on CA2 agar for 7 days at 20°C. In all cases, the colonies are spreading, but they still are rather small after 1 week. On diluted CA2 agar containing nitrate as the sole nitrogen source, the swarm colonies of all three strains remain rather delicate. In (e), small crystals can be seen within the colonies, as is often the case with lysobacters.

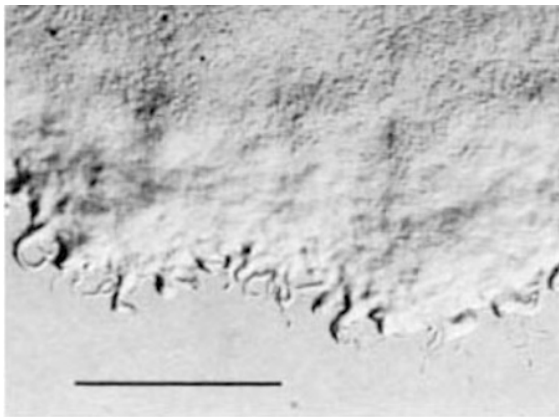
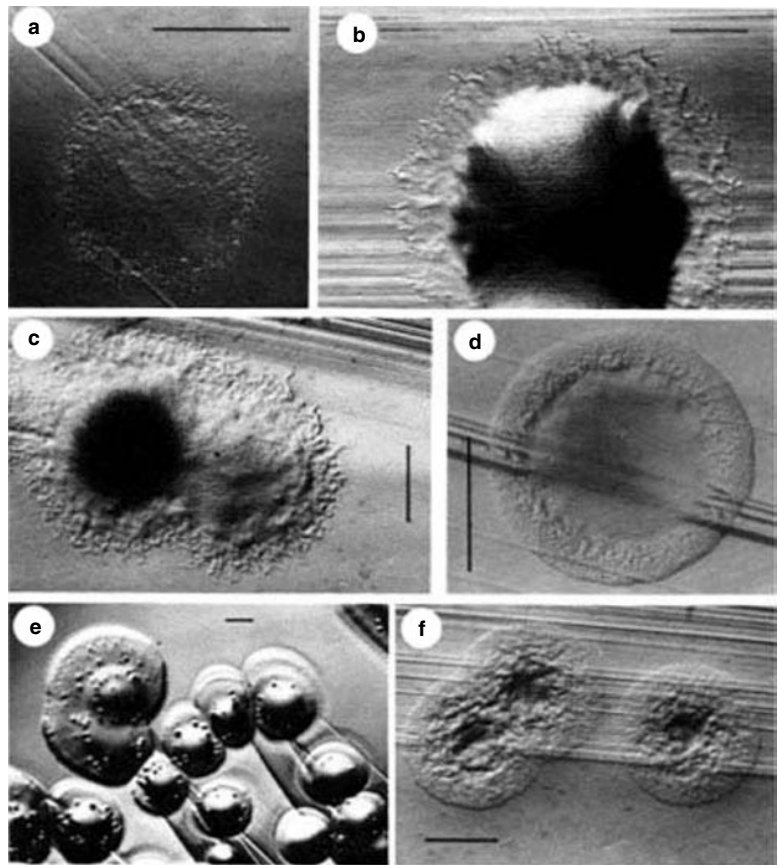


Fig. 4. *Lysobacter antibioticus* strain UASM L17. Edge of a swarm colony showing the typical flamelike protrusions. CY agar, 7 days at 20°C. Bar = 1 mm.

## Habitats

The lysobacters live in soil and fresh water and appear to be wide spread and in many places are rather abundant. They have been isolated from soils in the Netherlands (Veldkamp, 1955), Canada (Christensen and Cook, 1978; Katznelson et al., 1964; Peterson et al., 1966), the United States (Ensign and Wolfe, 1965), and

Scotland (Daft et al., 1975). In Scotland, they have been found in agricultural soils and sand dunes where the pH was neutral to slightly alkaline (pH 8.8) and not below pH 6. Population densities of up to 500 plaque-forming-units (PFU) per cm<sup>3</sup> were observed. This may seem very low, but the isolation method was designed only to detect organisms that lyse cyanobacteria (forming plaques in the cyanobacterial lawns), which may not be the case with all lysobacters. Also, the counts would be much reduced if most of the lysobacters are attached to particles. In soil, the lysobacters probably subsist by degrading various biomacromolecules and microorganisms other than cyanobacteria. In fact, lysobacters are known to decompose nematodes and different kinds of bacteria, as already mentioned. So, while their ecological niche is not really identified, the enzymatic equipment and lytic capabilities of the lysobacters suggest that they preferentially live in places rich in (recalcitrant) organic matter and microbial life.

Much more is known about the freshwater habitats of the lysobacters because there strains exist that can lyse living and healthy cyanobacteria and for this reason aroused much interest (see reviews by Stewart and Daft, 1976, 1977). The bacteria were found in fish ponds in Israel (Shilo, 1967; Shilo, 1970), in sewage plants in the United

States (Stewart and Brown, 1969, 1971) and Scotland (Daft et al., 1975), and in lakes, reservoirs, and rivers in Great Britain (Daft et al., 1973, 1975). This, of course, does not mean that those lytic strains are the only lysobacters living in fresh water, as lysobacters were also isolated from that source by other techniques, and the ability of these strains to lyse cyanobacteria was never specifically demonstrated (in Canada: Christensen and Cook, 1978; in Germany: H. Reichenbach and coworkers, unpublished observations).

In contrast to cyanobacterial viruses, the lytic lysobacters have a wide activity spectrum with respect to the cyanobacterial species attacked. Thus, strain FP-1 lysed 9 out of 11 species of unicellular and filamentous cyanobacteria whereas the green alga *Chlorella pyrenoidosa* and the chrysophyte *Prymnesium parvum* were completely resistant (Shilo, 1970). All Gram-negative bacteria tested were also lysed, but Gram-positive bacteria were not. Strain CP-15 disintegrated 21 out of 23 strains of cyanobacteria (Daft et al., 1973), and strains CP-1 to -4 among themselves lysed 29 out of 42 cyanobacteria from nine different genera (Daft and Stewart, 1971). In the latter case, there were slight differences among the four strains with respect to the spectra of cyanobacteria attacked, and also different strains of the same cyanobacterial species could vary in their sensitivity to lysis. All 16 British *Lysobacter* strains used in these studies were serologically related and may have belonged to one species (Stewart and Daft, 1976).

The population densities of lysobacters observed in British waters are usually low (Daft et al., 1975), but since lysobacters attach themselves to plankton and other particles, the counts may not reflect the true numbers. In lakes in summer and at the water surface, between 0 and 400 PFU/ml were found, and the numbers decreased rapidly with depth (to 1 m). But 94% of all samples contained lytic bacteria. At the height of cyanobacterial water blooms, 0.001 to 0.05% of all cultivatable bacteria were lytic, and in general the population densities of cyanobacteria and lytic bacteria correlated well, with a slight shift in the time of the maxima. Large differences in the cell numbers could occur in simultaneous samples taken from different sites in the same lake. This apparently was simply due to wind drift which caused the plankton to accumulate at certain places. In samples with particularly high densities of cyanobacteria, the numbers of lysobacters could drop substantially. As the lysobacters are strict aerobes, their decline in number under such conditions was explained by oxygen depletion during the night, but perhaps the bacteria only seem to disappear because there was now an increased opportunity for

attachment. In February, after 3 months of low phytoplankton density (and low temperature), the count of lytic bacteria fell to zero at the water surface, but some lysobacters still survived in the depth at the sediment-water interface. From there and from soils around the lakes, the repopulation of the water bodies may take place in spring. Another source for lytic bacteria was found in sewage works where in summer the counts in the final effluents were around 700 PFU/ml, and in the effluents from filter beds even as high as 1,300 PFU/ml. Also, in sewage plants, the numbers dropped during winter, in February to about 1% of the summer counts (Daft et al., 1975). Dense populations of lytic bacteria were found in the sand filters of a waterworks at a reservoir with blooming cyanobacteria. Up to 2,500 PFU/ml were found in the effluent, and 59,000 PFU/ml on the filter sand. The maximum in the open reservoir was 92 PFU/ml (Daft et al., 1973). No lysobacters were detected in water from underground springs (Daft et al., 1975).

While the lysobacters are able to lyse bloom-forming cyanobacteria, their low population densities make it questionable whether they really play a role in the control of natural water blooms. In field trials, at least  $10^5$  cells/ml were required to induce rapid lysis in cyanobacterial populations. In small bays at the edge of a reservoir, which were separated artificially from the main body and inoculated to a density of  $10^6$  bacteria/ml, there was clear evidence for lysis within 24 h at 13°C, and within 60 h, the *Microcystis* population was completely destroyed. In the lysing cell suspension the number of lytic bacteria rapidly declined, which again was explained by a lack of oxygen (Daft et al., 1973; 1975). In summary, it appears that cyanobacteria and lysobacters have similar growth requirements and simply coexist rather than prey upon one another. As the lysobacters can grow perfectly well as saprophytes, they may utilize material, including biomacromolecules, excreted by the cyanobacteria. In fact, they appear to interfere in a subtle way with the photosynthesis of the cyanobacteria and their excretion of dissolved organic carbon, reducing the rate of the former and stimulating the rate of the latter, and are able to rapidly assimilate excreted material, particularly certain amino acids, and to grow exclusively on it with a generation time of about 10 hrs (20°C). In a similar way they also exploit bloom-forming green algae like *Scenedesmus quadricauda*, which they cannot lyse at all (Fallowfield and Daft, 1988). Under conditions unfavorable for the cyanobacteria, the lysobacters also may lyse some of them, but they probably have little to do with the cyclic break down of water blooms in nature.



## Isolation

Two properties of the lysobacters may be used for their enrichment: their efficient hydrolytic exoenzymes and their gliding motility. As both attributes are also found with other organisms, the enrichment techniques are not entirely specific.

Christensen and Cook (1978) recommend enriching soil samples with chitin, ground mushrooms, or *Arthrobacter* cells, and then leaving them for at least one month. After this period, the soil is suspended in water and appropriate dilutions are spread on yeast agar.

### Yeast Agar

Baker's yeast	0.5%
Agar	1.5%

Adjust to pH 7.2 and autoclave.

After incubation, colonies arise that are surrounded by lysis zones, and their number increases substantially during the enrichment phase. Two types are usually observed: 1) pink-colored (*Lysobacter antibioticus*) and 2) cream-colored (*L. enzymogenes*). Rarely, also, an off-white gummy colony may appear (*L. gummosus*). On subcultivation, most of the cream colonies produce two colony variants: 1) dirty-white mucoid, and 2) yellowish nonmucoid. When water samples from lakes and rivers are used, yellow-brown colonies are obtained (*L. brunescens*).

As all lysobacters appear to degrade chitin (Christensen, 1989), one also could try to isolate them on chitin agar as described by Veldkamp (1955). He used 1% of finely powdered chitin suspended in a medium containing 0.1%  $K_2HPO_4$ , 0.1%  $MgSO_4$ , and 2% agar. He purified the chitin from shrimp shells by a very cumbersome procedure, so a simpler method is given here as well as two chitin media, which are used in our laboratory with excellent results in enriching for cytophagans and myxobacteria.

### Preparation of the Chitin Stock Suspension (Modified from Hsu and Lockwood, 1975)

Finely divided commercial chitin (e.g., from Fluka or Sigma) is suspended in concentrated (32%) HCl. For 40 g of chitin, approximately 400 ml of HCl is required, but sometimes more HCl (600–800 ml) has to be used. Within about 30 min, a relatively thin, blackish colloidal solution is obtained. The hydrochloric acid solution should never stand for more than 1 h at room temperature to avoid degradation of the chitin. The solution is poured into 2 liters of ice-cold water, upon which the chitin precipitates immediately as a pure white, fluffy material. It is collected on a separating funnel under suction and extensively washed. During these manipulations the material should never become dry, because drying makes it very difficult to resuspend. The washed precipitate is dialyzed against tap water for 12 to 24 h until the pH of the water

remains above at least 4.5. Then enough distilled water is added to the chitin suspension to give a slurry which is sufficiently thin to be pipetted. The pH is adjusted to 7.2 with KOH. The exact volume is determined, and the approximate chitin content of the suspension calculated from the amount employed in the beginning. The material is distributed in convenient aliquots in bottles, autoclaved, and stored at 6°C. If the precipitated chitin appears too coarse, which may happen if the starting material was not well ground, it can be further homogenized with a blender.

Chitin agars are prepared best as overlay media, which saves chitin and gives clearer results, because the layer to be hydrolyzed is thinner and the material remains at the top of the plate, so that the bacteria have an easier access to it.

### CT6 Agar

#### Top layer:

$MgSO_4 \cdot 7H_2O$	0.1%
$K_2HPO_4$	0.02%
Agar	1.5%

Adjust the pH to 7.5. After autoclaving, enough of the sterile chitin suspension is added to give a good turbidity, which should be achieved with about 0.5% chitin and not more than 30% (by volume) of the suspension. The medium is poured as a thin layer on top of the following base agar:

#### Base agar:

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

Adjust the pH to 7.2 and autoclave.

### CT7 Agar

The top layer is as above; the base agar, however, is water (WAT) agar, which is as follows:

#### WAT agar:

$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

Adjust the pH to 7.2 and autoclave.

In CT7 agar, chitin is the only nitrogen, carbon, and energy source besides agar, and thus the medium is more selective. But not all chitin degraders grow on it. On CT6 agar, on the other hand, chitin degradation is sometimes suppressed by the peptone, and organisms that are not chitinovorous also grow on it.

Lysobacters decompose many bacteria, living and dead, and therefore may be isolated on such substrates. Strain AL-1 was obtained from lysis zones in lawns of *Arthrobacter crystallopoietes* (Ensign and Wolfe, 1965). Aqueous soil extracts were streaked on plates of medium A and incubated at 30°C.

**Medium A**

A basal agar with 0.5% peptone and 1% agar was overlaid with the same medium containing  $10^9$  cells/ml of *Arthrobacter*.

The bacteria from the plaques that developed in the lawn were purified by plating diluted suspensions on medium B.

**Medium B**

Agar, 1.5%, containing  $10^9$  cells/ml of washed *Arthrobacter*.

Presumably the *Arthrobacter* cells were living in the isolation medium and dead in the purification medium. Strain AL-1 was also maintained on medium B (at 30°C, with weekly transfers).

Similar isolation techniques may be devised with other bacteria and in fact have been used repeatedly to obtain lysobacters that lyse cyanobacteria. Stewart and Brown (1969) and Shilo (1970) isolated their organisms from water collected from a waste stabilization pond and a fish pond with a water bloom, respectively. Both groups used the soft-agar overlay technique developed for the isolation of cyanophages, and living *Nostoc muscorum* or *Plectonema boryanum* as the indicator organisms. Shilo (1970) first enriched the lytic agent by inoculating the sample into a *Plectonema* culture. While viral plaques appear after just 2–3 days, the bacterial plaques need 5–7 days to develop, and in addition are slightly sunken into the agar. Further, the bacteria also produce lysis zones in layers of autoclaved cyanobacteria. Shilo (1970) separated her strain from the cyanobacteria by transfer to a mineral salts liquid medium with 0.2% Casitone and incubation in the dark. The overlay technique may also be used for counting the lytic bacteria. Stewart and Brown (1969) determined for strain N-5 (myxobacter 44) a ratio of 1.3 for the number of viable cells to plaque-forming units.

A slightly different method was applied by Daft and Stewart (1971) and Daft et al. (1973). The cyanobacteria were grown on a suitable mineral salts medium with 0.6% agar. On the lawns produced after 7 days at 22–25°C, 1-ml water samples from the top-most layer of lakes or other surface waters, preferentially such with a cyanobacterial water bloom, were spread and the lysobacters isolated from the lysis zones that arose in the lawns. As indicator organisms, *Nostoc ellipso sporum*, *Anabaena catenula*, *A. flos-aquae*, or *Phormidium foveolarum* were used, and *N. ellipso sporum* (strain 1453/19 Cambridge University) proved particularly sensitive to lysis. The CP strains could be maintained without loss of lytic activity on CP agar.

**CP Agar**

K <sub>2</sub> HPO <sub>4</sub>	17 mg
MgCl <sub>2</sub> · 6H <sub>2</sub> O	49 mg
CaCl <sub>2</sub> · 6H <sub>2</sub> O	15 mg
NaCl	60 mg
FeCl <sub>3</sub>	0.3 mg
EDTA	7.4 mg

Standard trace element solution\*

Casitone (Difco)	2 g
Agar	6 g
Distilled water	1 liter

The mineral part is that of ASM medium (Daft and Stewart, 1971) for the cultivation of algae (and probably dispensable for the lysobacters).

\*See, for example, Drews (1974), p. 6.

Lysobacter colonies may be recognized by their spreading growth due to gliding motility, and colony morphology can thus be used as a lead to isolate the organisms. Thus, on plates designed for the isolation of myxobacteria and consisting of water agar (WAT agar) with streaks of living *Escherichia coli* that are inoculated with soil, we occasionally also observe swarming lysobacters.

The purification of *Lysobacter* strains is easy and is done either by plating of diluted cell suspensions or by making transfers from the advancing edge of swarm colonies. As swarming is much reduced or completely suppressed on rich media, the latter possibility exists only when the bacterium is grown on a lean medium like yeast agar or CY agar (see below). To avoid confluence of the arising colonies, plating is done best on a rich medium such as standard nutrient agar (Difco).

**Cultivation**

The lysobacters are robust, adaptable organisms, and their cultivation is no problem. They actually grow on any bacteriological standard medium. Christensen and Cook (1978) recommend the three following media:

**PC Agar (Plate Count Agar, Difco)**

Yeast extract	0.25%
Tryptone	0.5%
Glucose	0.1%
Agar	1.5%

**CC Agar (Cook's *Cytophaga* Agar; Christensen and Cook, 1972)**

Tryptone	0.2%
Agar	1.0%

**SA Agar (Skim-Milk Acetate Agar; Christensen and Cook, 1972)**

Skim milk powder	0.5%
Yeast extract	0.05%
Sodium acetate	0.02%
Agar	1.5%

The pH for all of these media is adjusted to about 7.0 and the medium is sterilized by autoclaving. On PC agar, the colonies remain small and compact like those of nongliding bacteria, whereas on CC and SA agar, spreading swarms are obtained. Another good medium for lysobacters, including stock cultures, is the Yeast Agar mentioned above. On this medium the organisms form thin, spreading swarms, and because no or much less ammonia is produced than on the peptone media, the cultures tend to remain viable for longer time.

SA medium may also be used as a broth for liquid cultures, but a nonturbid medium is more convenient, e.g., 1% yeast extract medium (Ensign and Wolfe, 1966). We often use PEP medium.

#### PEP Medium

Peptone from casein, tryptically digested	1%
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.1%

Adjust the pH to 7.2 and autoclave.

Other peptones can be used as well, e.g., Casitone (Difco), which is a pancreatic digest of casein, and casamino acids have also been employed successfully (Gillespie and Cook, 1965). *L. brunescens* appears not to grow well or to lyse early in pure peptone media. It could, however, be cultivated with a generation time of 4 h at 20–22°C in tryptone-starch medium.

#### Tryptone-Starch Medium (von Tigerstrom and Stelmaschuk, 1987b)

Tryptone	0.8%
Yeast extract	0.2%
Starch	0.3%
MgCl <sub>2</sub>	0.02%

A fully defined medium for *L. enzymogenes* strain AL-1 was developed by Ensign and is mentioned in Tan et al. (1974):

#### Defined Liquid Medium (for Strain AL-1)

Aspartic acid	0.2%
K <sub>2</sub> HPO <sub>4</sub> · 3H <sub>2</sub> O	0.34% (0.03 M)
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.1%
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.01%
Tap water	1%
Glucose	0.5%

Adjust to pH 7.0 and autoclave.

Although lysobacters tolerate relatively high pH values and can grow above pH 10 (Christensen and Cook, 1978), their optimum is between pH 7 and 9. While all appear to grow at 30°C, their temperature optimum is some-

times lower, and cultivation at 25°C or even 22°C may give better results. The organisms are strict aerobes, and liquid cultures have to be shaken.

The generation times are typically around 2.5 h (2.3 h for *L. enzymogenes* strain 495 in 0.8% yeast extract broth at 22°C: von Tigerstrom and Stelmaschuk, 1989; and 2.6 h for strain myxobacter 44 in a mineral salts medium with 0.2% starch and 0.25% peptone, 30°C: Stewart and Brown, 1971. Much longer generation times of 10.7 and 12.6 h are reported for growth under quasi-natural conditions in nutrient-poor culture filtrates from cyanobacteria and green algae, respectively (strain CP-1, 20°C: Fallowfield and Daft, 1988). A maximum cell yield of 2.5 g/l (dry weight) was obtained with strain 495 in condensed fish solubles with 2% glucose (Wah-On et al., 1980).

Large-scale fermentations with lysobacters have been performed for the production of the antibiotic myxin, and of an enzyme complex capable of lysing living yeast cells. While apparently nothing has been published about the industrial process developed by Hoffmann-La Roche (Nutley, NJ) for the manufacture of myxin with strain "*Sorangium*" 3C, some data on 10-liter fermentations are given in a Canadian patent (Can. Pat. 784,213; 30 April 1968). The fermentation was performed in the following medium: Tryptone, 0.1%; glucose, 0.1%; K<sub>2</sub>HPO<sub>4</sub>, 0.1%; MgSO<sub>4</sub>, 0.02%; CaCl<sub>2</sub>, 0.01%; and FeCl<sub>3</sub>, 0.001%; at pH 7.5 and 25°C. The aeration rate was 0.1 liter air per liter medium and min; the stirring rate, 300 rpm; the harvest time, 20 h with a 2% (v/v) inoculum, and 12 h with a 10% inoculum.

Production of yeast-lytic enzymes by "*Cytophaga*" NCIB 9497 was studied at up to a 900–1 liter scale (Asenjo et al., 1981). The medium consisted of 1% yeast extract and 1% glucose and permitted a maximum growth rate ( $\mu_{\max}$ ) of 0.36 h<sup>-1</sup>. The inoculum size was 3.8%, the initial pH 7.2, and the temperature 26°C. The aeration rate was set at 0.2 liter air per liter medium and min, and the impeller tip speed between 1.21 and 1.57 m·sec<sup>-1</sup> (around 100 rpm). It appeared that a relatively low level of dissolved oxygen around 20–30% was favorable, particularly in the beginning. Polypropylene glycol 2000 was added as an antifoam agent and was well tolerated at 2 ml/l but not higher. Still, after 24 h, the broth began to foam heavily. Harvest was at 30 h with a good yield of active enzyme, part of which was set free only by cell lysis at the end of the fermentation. Early cell lysis may be a serious problem. Obviously it is essential to start with young, vigorous seed cultures, e.g., from freeze-dried material.

## Preservation

Stock cultures may be kept on yeast agar at room temperature (21°C) and should be transferred every 1 to 2 weeks. With stock cultures on peptone media, production of toxic levels of ammonia can be a problem. Storage of cultures in the cold is not recommended. In one reported case, all cells were dead after 3 weeks at 5°C (Asenjo et al., 1981). It seems understandable that the lytic enzymes produced by lysobacters also destabilize resting cultures.

Lysobacters survive freezing very well, either at -80°C or in liquid nitrogen. Cells from young plate cultures are suspended in PEP medium, or young shake cultures in the same medium are taken, and 1-ml samples are frozen without further precautions. The preserved cultures are reactivated by immersing them in tap water for a quick thawing and transferring the content as soon as it is liquefied. The longest storage period we have tested so far was 13 years at -80°C, which was reliably tolerated by all strains.

Lysobacters also can be lyophilized in skim milk by the standard procedure. It is reported that reactivated freeze-dried cultures may show a slightly different colony morphology and reduced bacteriolytic and proteolytic activity (Christensen, 1989). We dry the organisms with good results at room temperature, which may be less harmful to them. A few drops of a cell suspension in sterile skim milk are applied to a plug of lyophilized skim milk in an ampule. The wetted plug is then dried in vacuo, the ampule filled with nitrogen gas, and sealed.

## Characterization

Morphologically, the various lysobacter species resemble one another closely (Christensen and Cook, 1978; Christensen, 1989). The cells are slender cylinders with rounded ends, and, at least in young healthy cultures, are rather regular in shape (Figs. 1 and 2). They typically measure 0.3–0.6 × 2–6 μm, but much longer threads, up to 70 μm, are usually also present. Those filaments obviously are incompletely divided cells and cell chains. A mixed population of short and long cells is very characteristic for the lysobacters and distinguishes them at once from any myxobacterium. To avert confusion, it should be mentioned that the morphological description given by Veldkamp (1955) of his chitinolytic strain does not fit the present NCIB 8501 strain, which indeed is a typical lysobacter. Instead, Veldkamp has given the morphology and cyclic-shape change of *Flexibacter filiformis*, which also is strongly chitinolytic.

Electron micrographs of thin sections show a typical Gram-negative cell wall, large mesosome-like structures in the center or at the poles, and granules interpreted as poly-β-hydroxybutyrate and polyphosphate. On the surface there may be ruthenium-red-positive material, presumably slime (Shilo, 1970; Stewart and Brown, 1971; Stewart and Daft, 1977); in other cases no such material was seen (“myxobacter” AL-1: Pate and Ordal, 1967). True rhabdosomes, i.e., the contracted tails of a defective phage (Reichenbach, 1967), were found in cell lysates of “*Sorangium*” 495 (Pate et al., 1967).

In contact with an interface, the lysobacters may move by gliding. The movements usually are rather slow but observable under the microscope. An average speed of 1 μm per min (30°C) has been reported (Veldkamp, 1955). When suspended in liquid the cells are nonmotile.

On lean media like CC, SA, and yeast agar, most lysobacters produce thin, filmlike swarms with little flamelike extensions at the edge (Figs. 3 and 4). Only *L. gummosus* appears not to spread on any medium, and its gummy colonies always show an entire edge. In general, the spreading of lysobacter swarms is not too fast, and it may take 2–3 weeks before they cover the entire plate. The swarm sheet itself may be somewhat slimy, but otherwise it is rather unstructured and smooth. This distinguishes the lysobacter swarms from myxobacterial swarms, which almost always are morphologically differentiated with radial veins, concentric ridges, and oscillating waves or ripples, and which in addition may etch themselves deeply into the agar surface (degenerate strains may produce homogeneous, slimy swarms). On rich media like PC agar, the colonies tend to remain compact, small, with a smooth convex surface and an entire edge. Those colonies often are highly mucoid. Enough slime may be produced to drop from the colony into the lid of the inverted dish (Ensign and Wolfe, 1965). As briefly mentioned, *L. enzymogenes* produces two different colonies, one dirty-white and mucoid, the other yellowish and nonmucoid. While both colony types are always obtained when starting from white colonies, the yellow ones yield only the yellow type. Physiologically and biochemically, the two organisms are identical (Christensen and Cook, 1978). Often, small, colorless crystals are seen within the colonies (Fig. 3e). In the case of *L. gummosus*, the colonies are of a rubbery consistency.

The color of colonies of *Lysobacter* may be off-white; cream; pale to deep yellow, sometimes with a greenish hue; pinkish; salmon; or orange-brown, depending on the species and on the medium. The chemical nature of those pigments is not known. The published absorption spectra of crude extracts show maxima or shoulders at

455, 482, and 516 nm (*n*-hexane) for salmon-colored strain FP-1; 448, 466, and 470 nm (ethanol) for three yellow "myxobacters" (Stewart and Brown, 1971); and 424, 442, and 464 nm (in methanol) for the yellow strains CP-1 and CP-15 (Daft and Stewart, 1971; Daft et al., 1973). While those spectra suggest a polyene chromophore, perhaps a carotenoid, they may be spectra of a mixture of pigments and thus cannot be reliably interpreted. Many strains produce a water-soluble, dark-brown, probably melanoid pigment, which is particularly prominent in old cultures and on media containing amino acids or peptones. Color reactions of phenolic compounds indicate the presence of a mono- and diphenol oxidase (Stewart and Brown, 1971). Deep-red crystals of myxin may appear within the colonies of *L. antibioticus*. Also, many cultures give off an unpleasant *Pseudomonas*- or pyridine-like odor.

In agitated liquid media, the lysobacters grow as homogeneous cell suspensions. When gently rotated, those cultures appear silky. Depending on the medium, liquid cultures may become somewhat viscous, and those of *L. gummosus* virtually solid.

Relatively little has been published about the composition of the lysobacter cell. A typical Gram-negative peptidoglycan was demonstrated in *L. enzymogenes* strain AL-1, with meso-diaminopimelic acid and D-alanine cross-bridges (Harcke et al., 1975). The inner and outer membrane could be separated starting from osmotically shocked cells. The outer membrane had a density of 1.30 g·cm<sup>-3</sup> (CsCl), the inner one of 1.23 g·cm<sup>-3</sup> (Hartmann et al., 1977). Ubiquinone Q-8 is the only respiratory quinone of *L. antibioticus* and *L. enzymogenes* (M. D. Collins, personal communication). This allows one to quickly distinguish lysobacters from myxobacteria as well as from organisms of the *Cytophaga* group (including high GC strains of *Taxobacter*) which all contain menaquinones exclusively. The ribosomes of "myxobacter" 495 (*L. enzymogenes*) were difficult to isolate because tightly bound nucleases and proteases were attached to them and could not be removed by the usual methods (Sendecki et al., 1971). Otherwise, lysobacter ribosomes have the typical prokaryotic composition. The rRNA has the same base ratio as that of *E. coli* (GC/AU = 1.20). The presence of poly-β-hydroxybutyrate in the lytic "myxobacters" was substantiated by its conversion into crotonic acid (Stewart and Brown, 1971). The compound accumulates in the course of cultivation, with a maximum on the third day. Capnoids, a new type of sulfonolipids found in bacteria of the *Cytophaga* group (Godchaux and Leadbetter, 1983), and multicopy single-stranded DNA common in myxobacteria

(Dhundale et al., 1985), are both absent in lysobacters. The lysobacter DNA has a high GC content of 65–71 mol% (Christensen and Cook, 1978; Daft and Stewart, 1971; Mitchell et al., 1969; Shilo, 1970; Stewart and Brown, 1971).

A number of metabolic enzymes have been studied in *L. enzymogenes* strain AL-1 (Guntermann et al., 1975; Hartmann et al., 1977). In the cytoplasm there is an α- and a β-glucosidase, a β-galactosidase, and an isocitrate dehydrogenase; on the cytoplasmic membrane, a succinate dehydrogenase; and bound to the outer membrane, an alkaline phosphatase and a *N*-acetyl glucosaminidase. The activities of those enzymes change during the cell cycle. Two distinct patterns were noted, one for the five hydrolases, the other for the two dehydrogenases. In the case of the α-glucosidase, the activity increase was shown to be due to de novo enzyme synthesis. While β-glucosidase and β-galactosidase are constitutive, α-glucosidase is inducible by maltose. In a study on the phylogenetic implications of the isozyme pattern of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (important for the synthesis of aromatic amino acids) in superfamily B (to which *Lysobacter* belongs according to 16S rRNA data), *L. enzymogenes* was found to possess only one enzyme variant, the one that is sensitive to feedback inhibition by tryptophan and, unique for *Lysobacter*, ultrasensitive to chorismate (Ahmad et al., 1986). This pattern is shared only by group V pseudomonads, which fits its classification based on 16S rRNA studies. In addition, the lysobacters produce a host of exoenzymes. As some of those are of a more general interest and have found applications, they will be discussed under "Practical Aspects," this chapter.

For a large number of strains, comparative physiological and biochemical tests were performed and used for the differentiation of species (Christensen, 1989; Christensen and Cook, 1978). The strains studied for their ability to destroy cyanobacteria were not characterized in the same scheme as those able to destroy other bacteria, so that the following generalizations may not always apply to them.

The lysobacters are aerobic organisms, although many strains appear to grow best at a reduced oxygen level (10% O<sub>2</sub>). In the oxidation-fermentation (OF) test with glucose, practically all strains show oxidative growth but many also show fermentative growth. Growth of the lytic freshwater organisms stops immediately when the culture is flooded with N<sub>2</sub> gas; no growth is obtained in paraffin-sealed tubes, and the generation time increases to 9 h (30°C) when the oxygen tension is reduced to 20% of the normal atmospheric pressure (Daft et al., 1975; Stewart and Brown, 1971). Catalase and oxidase

are positive, but the catalase reaction is very sensitive to the culture conditions and sometimes is very weak or even absent (H. Reichenbach, unpublished observations).

The pH range is 4.5 to over 10, the optimum between 7 and 9. There is almost always a remarkable tolerance to alkaline conditions. Even the freshwater strains usually grow well at pH 9 (Stewart and Brown, 1971; Daft et al., 1975). The ability to grow at an acid pH varies with the different strains; many do not grow below pH 6. During growth on peptones, the pH may rise substantially due to ammonia production.

The temperature optimum is usually around 30°C, even for the freshwater organisms, but it varies substantially from isolate to isolate. It may be as low as 25°C or as high as 40°C. A few strains can grow at 2°C and at 50°C, but the limits often are 4 and 40°C.

The salt tolerance is usually limited to 1% NaCl or less, and no strain was found to grow in the presence of 3% NaCl.

The lysobacters are chemoorganotrophs. For many strains, NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, glutamate, or asparagine serve as the sole nitrogen source whereas urea appears to be utilized only by a few strains. Some organisms, however, are more fastidious. Several of the strains that lyse cyanobacteria do not grow on inorganic nitrogen (Daft et al., 1973; Shilo, 1970), while others do (Stewart and Brown, 1971). All lysobacters grow on peptone as the only organic substrate, but the type and concentration of the peptone may be critical. Casitone (Difco) seems to be suitable in all cases, but strain FP-1 has a low optimum of 0.2% for Casitone and does not grow at 0.5%. The same strain also does not accept yeast extract, tryptone, or casamino acids, and does not grow on nutrient broth or nutrient agar (Shilo, 1970). In general, peptides appear to be the preferred carbon and energy source, and monosaccharides may only be used slowly (von Tigerstrom and Stelmaschuk, 1987b).

Glucose is probably utilized by all strains. Growth may take place on a wide spectrum of carbohydrates, e.g., in one case on: glycerol, mannitol, arabinose (weak), glucose, galactose, fructose (weak), mannose, lactose, sucrose, raffinose, and rhamnose (Veldkamp, 1955). Acid is produced from glucose and from several other sugars in a varying pattern that may be useful for the differentiation of species. Many strains grow on citrate as the sole carbon source.

All strains are strongly proteolytic as can be seen, e.g., by cultivation on skim milk agar. They liquefy gelatin, peptonize milk, and produce  $\alpha$ -,  $\beta$ -, or  $\gamma$ -type hemolysis on sheep blood agar, including strain AL-1, which originally was reported to be negative for hemolysis (Ensign and Wolfe, 1965). Almost all lysobacters hydro-

lyze chitin. Veldkamp (1955) observed *N*-acetylglucosamine, glucosamine, acetic acid, and NH<sub>4</sub><sup>+</sup> as products. Several of the isolates that decompose cyanobacteria did not attack chitin (the failure was perhaps due to the method used; Stewart and Brown, 1969; Daft et al., 1975), while others do (Stewart and Brown, 1971). Crystalline cellulose (filter paper) is not decomposed, but many strains degrade carboxymethyl cellulose, and at least some strains can even grow on it as the only organic substrate (Stewart and Brown, 1971). While agar is not liquefied or softened, gelase fields (uncolored areas) may appear upon flooding with iodine solution (Shilo, 1970). Starch, pectate, and alginate may or may not be decomposed. Many strains produce lipases that cleave Tweens.

Ammonia is produced from proteins and peptones. Nitrate is only rarely reduced to nitrite. Some strains generate H<sub>2</sub>S. The indole, methyl red, and Voges-Proskauer tests are always negative, and the phosphatase test is always positive.

Sodium dodecyl sulfate usually diminishes growth at 0.01% and completely inhibits it at 0.1%. All lysobacters appear to be sensitive to polymyxin B, although the result may vary somewhat with the way the test is done (Christensen and Cook, 1978; Shilo, 1970; Stewart and Brown, 1971). This is a valuable distinguishing characteristic in so far as most *Cytophaga*-like bacteria are resistant to polymyxin B (Mitchell et al., 1969). All strains of *L. brunescens* as well as the freshwater lysobacters (Shilo, 1970; Stewart and Brown, 1971) also are rather sensitive to actinomycin D (around 2  $\mu$ g/ml), which is in contrast to most Gram-negative bacteria but in accordance with the behavior of most other gliding bacteria (Dworkin, 1969). All *L. brunescens* strains are inhibited by chloramphenicol, penicillin G, and usually also streptomycin, but most other lysobacters are not. Strain FP-1 was also resistant to erythromycin and tetracycline, but inhibited by kanamycin and neomycin (Shilo, 1970). The strains of Stewart and Brown (1971) and of Daft et al. (1975) were sensitive to all these and several more antibiotics.

As already mentioned, the lysobacters are able to kill and disintegrate many living and healthy microorganisms, such as both Gram-positive and Gram-negative bacteria, including actinomycetes and cyanobacteria; filamentous fungi; algae; and even nematodes. Gram-negative strains are usually less readily killed and lysed than Gram-positive ones. The first signs of lysis may be observed within minutes after the culture broth has been added to a suspension of sensitive bacteria (Gillespie and Cook, 1965). The technique by which the lysobacters destroy cyanobacteria is particularly well documented

(Daft and Stewart, 1971, 1973; Daft et al., 1973, 1975; Shilo, 1970; Stewart and Brown, 1970). They use two different mechanisms: 1) One strain, strain N-5 (myxobacter 44) attacks the cyanobacteria with free exoenzymes (Stewart and Brown, 1969, 1970, 1971). The first assault seems to be on the cell wall with lysozyme-like enzymes, but later the whole cell with the exception of the membranes is digested. Heterocysts and akinetes are more resistant but finally also destroyed. Only their walls remain. 2) All other strains need to be in contact with the cyanobacteria before they can lyse them (Daft and Stewart, 1973; Shilo, 1970). Since neither culture supernatants nor cell extracts are sufficient to disintegrate cyanobacteria on solid or in liquid media, enzymes bound to the surface of the lysobacter cell are probably involved. It appears that the lysobacters move actively toward their prey, perhaps attracted by the oxygen produced by photosynthesizing cells. They attach themselves with one cell pole often close to the cross-septa of the filaments, and then become fixed perpendicularly on the surface of the target cells. No special organelle for attachment is recognizable. The need for contact explains why lysis does not take place in shaken cultures. In unshaken liquid cultures, the lysobacters are found fixed to the cyanobacteria within 20 min after mixing. A single lysobacter cell may lyse a *Nostoc* cell within 20 min. It then moves on and attacks another cell in the filament. There is no specific order in which the cells along a filament are assaulted. When a lysobacter culture of a sufficiently high cell density is added to a cyanobacterial population, lysis may be complete within 1 to 3 h. The time required depends, of course, on environmental factors, notably oxygen and temperature. Thus, in a well-aerated culture at 26°C, *Nostoc ellipsoforum* is completely destroyed within 60 min. On ultrathin sections in the electron microscope, it can be seen that the first structure to disappear is the peptidoglycan layer. The cell contents are then gradually broken down, and only the cell membranes, lipid droplets adhering to them and, if present, the gas vacuoles remain. The latter rise to the surface where they can be collected. The membranes often coil up, looking in cross-sections like scrolls. Protoplasts may be released from the filaments. If there is an outer sheath, it also may disintegrate. In the case of *Oscillatoria redekei*, amorphous material disappears from the sheath and a fibrous layer is left. Heterocysts initially resist lysis but eventually also disintegrate, either under the impact of the bacterial enzymes or by autolytic processes started after the cells are released from the filaments. In infected cyanobacterial cultures, the chlorophyll *a* content and the nitrogenase activity go down concomitantly with lysis, so that

either parameters can be used to measure its progress (Daft et al., 1973).

Tan et al. 1974 have devised a method to establish synchronized cultures of myxobacter AL-1: Cells of different sizes, equivalent to different stages of the growth cycle, were separated by centrifugation in a sucrose density gradient. As already mentioned, in this way, changes of enzyme activities during growth could be determined.

## Taxonomy and Identification

The first step in the identification procedure is to make sure that an isolate belongs to the genus *Lysobacter*. An organism qualifies as a lysobacter if it is a chemoorganotrophic, aerobic, unicellular, gliding, Gram-negative bacterium with a GC content of 65 to 71 mol%. Those characteristics are, however, shared by the myxobacteria and by certain organisms of the *Cytophaga* group (such as *Taxeobacter*). Some myxobacteria have a very similar cell shape (for instance, *Polyangium*, *Sorangium*, and *Chondromyces*) but the populations never show the variation in cell length typical for lysobacters. The morphology and structure of myxobacterial swarms is usually completely different, myxobacteria produce fruiting bodies and myxospores, and they contain menaquinones instead of ubiquinones as respiratory quinones. The organisms of the *Cytophaga* group, as far as they are gliding, may form very similar soft, slimy swarm colonies, but their cells usually look much different, they often contain flexirubin-type pigments and always menaquinones. The colonies of most *Cytophaga*-like bacteria are more-or-less yellow to orange, often brightly colored, particularly on peptone agar, and turn red if covered with 20% KOH (flexirubin reaction), which is never observed with lysobacter strains. Unfortunately, *Taxeobacter*, the only genus that in its GC content comes close to *Lysobacter*, is red. It has a similar cell shape to *Lysobacter*, but its cells are much stouter and tend to arrange themselves side-by-side in a palisade like fashion. Also, *Taxeobacter* lacks the unpleasant smell of *Lysobacter*.

Presently, four *Lysobacter* species are recognized. Their differential characteristics are listed in Table 1. The separation of the species rests entirely on physiological and biochemical data and is based on phenotypic analysis of a large number of strains (Christensen and Cook, 1978). However, the relatively wide GC range of the strains of two species (65–70 mol% in *L. enzymogenes* and 66–69 mol% in *L. antibioticus*) and the substantial differences in the temperature maxima of strains of *L. brunescens* (37–50°C) suggest that more than one taxon may

Table 1. Characteristics distinguishing the four species of *Lysobacter*.

Character	<i>L. enzymogenes</i> <sup>a</sup>	<i>L. antibioticus</i>	<i>L. brunescens</i>	<i>L. gummosus</i>
Colony morphology	Sheetlike, soft slimy	Sheetlike, soft slimy	Thin, filmlike	Compact, gummy
Colony color	Cream to greenish-yellow	Pink to greenish-yellow	Yellow to brown	Yellowish-gray
Viscosity of broth cultures	+	+	-	+++
Urea as sole N-source	V	-	-	+
Citrate as sole C-source	+	+	+	-
Acid from cellobiose	+	+	-	+
Acid from sucrose	+	+	-	+
Acid from lactose	+	V	-	+
Hydrolysis of carboxymethyl cellulose	+	+	-	+
Hydrolysis of pectate	+	-	+	+
Hydrolysis of starch	V	-	+	-
Hemolysis (sheep blood)	$\alpha$ , $\beta$ , or $\gamma$	$\beta$ or $\alpha$	$\gamma$ or -	$\alpha$
Sensitive to chloramphenicol	V	-	+	-
Sensitive to actinomycin D	V	-	+	V
GC content, T <sub>m</sub> (mol%)	65-70	66-69	67	66
Habitat	Soil	Soil	Fresh water	Soil
ATCC no. of type strain	29487 <sup>b</sup>	29479 <sup>c</sup>	29482	29489

Symbols: +, present; -, absent; V, variable.

<sup>a</sup>Type species of the genus *Lysobacter*.

<sup>b</sup>Formerly "*myxobacter*" or "Sorangium" 495. <sup>c</sup>Formerly "Sorangium" 3C. Adapted from Christensen (1989) and Christensen and Cook (1978).

be hidden among the strains presently allocated to one species. First attempts to subdivide one of the species have been made (*L. enzymogenes* subsp. *enzymogenes* and subsp. *cookii*; Christensen and Cook, 1978), but before that can reasonably be done, the methods of molecular taxonomy, notably DNA-DNA hybridization, need to be used. It seems doubtful whether the occurrence of myxin can be used as a characteristic of the species *L. antibioticus*, because production of an antibiotic usually is strain- and not species-specific.

As there is a free exchange of organisms between soil and fresh water, the habitats given in Table 1 for the various species should not be taken as absolute. In fact, the "freshwater" lysobacters attacking cyanobacteria have also been found in soil (Daft et al., 1975), and we have isolated myxin-producing lysobacters from freshwater (H. Reichenbach, unpublished observations).

Two more species not shown in Table 1 also have been proposed *L. lactamgenus* (Ono et al., 1984) was studied because of its new cephem antibiotics, the cephabacins. While several characteristics of the strain are in accordance with a lysobacter, it differs substantially in its extremely high GC content (76 mol%, T<sub>m</sub>) and in its inability to produce acid from any sugar. *L. albus* (Nozaki et al., 1987) synthesizes the novel antibiotic lactivicin. The description of this isolate is very preliminary and lacks essential data, e.g., on gliding motility and GC content. Acceptance of the new species will require that the strains be made freely accessible to others in the field.

## Practical Aspects

The spectacular lytic capabilities of the lysobacters suggest that they may play an important role in the control of microbial populations in nature, although their population densities appear never to become high enough to cause a breakdown of a cyanobacterial water bloom (Daft et al., 1975). Still, it may be possible to eliminate blooming organisms in a limited area, or pathogenic or otherwise undesirable bacteria in sewage plants, like the filamentous organisms responsible for the bulking of sludge, by introducing lysobacter strains with strong, lytic enzymes. The potential is probably limited by the requirement for cell-to-cell contact as a prerequisite for lysis, which would restrict any application to nonagitated environments, such as trickling filters or dense, floating mats of the target organisms.

The ability of lysobacters to synthesize new secondary metabolites is of particular importance. The following antibiotics have been isolated from lysobacters (and *Lysobacter*-like organisms): the phenazine-*N*-oxide, myxin (Peterson et al., 1966; Weigle and Leimgruber, 1967); two cyclic decapeptides, myxosidin A and B (Clapin and Whitaker, 1976, 1978 Monahan and Whitaker, 1976); the quinoline compound, G 1499-2 (Evans et al., 1978); new cephem antibiotics, the cephabacins (Harada et al., 1984; Ono et al., 1984); the acyltetramic acids, catacandin A and B (Meyers et al., 1985); a dicyclic dipeptide with lactam-like properties, lactivicin (Harada et al., 1986, 1988 Nozaki et al., 1987, 1989); and a



dibasic macrocyclic peptide lactone, lysobactin (Bonner et al., 1988; O'Sullivan et al., 1988; Tymiak et al., 1989).

Of those antibiotics, myxin has been produced industrially by Hoffmann-La Roche in Nutley for some time. It was applied in the form of a copper chelate called cuprimyxin and sold under the trade name Unitrop®. In contrast to free myxin, the chelate is very stable and practically insoluble. In contact with animal tissue, myxin is gradually released, so that the complex guarantees a sustained activity. Cuprimyxin was applied topically, mainly against skin infections including dermatophytes, and its use was restricted to veterinary medicine. The antibiotic is produced by strains of *L. antibioticus*, which was originally named "*Sorangium*" 3C (in the chemical literature, it is often further mislabelled as "*Sporangium*"). Myxin acts on DNA. There exists a voluminous literature on the various aspects of myxin, which can, however, not be reviewed here (see, e.g., on the chemistry: Sigg and Toth, 1967; Weigele and Leimgruber, 1967; Weigele et al., 1971; on the mechanism of action: Behki and Lesley, 1972; Lesley and Behki, 1967; on the biological activity and application: Grunberg et al., 1967; Maestrone and Brandt, 1980; Maestrone and Mitrovic, 1974; Maestrone et al., 1972; McDonald et al., 1980; Snyder and Imhoff, 1975).

The main reason why the lysobacters initially were noticed was their rich stock of exoenzymes. Several of those enzymes have a potential for an application in research or industry. One of the *Lysobacter* proteases has been studied in great detail for theoretical reasons and is one of the best-understood enzymes. Again, the literature on this topic is by far too extensive to be reviewed here comprehensively (see below).

Strain NCIB 8501 (if the present strain is really identical with the one first described, see above) was the first lysobacter to be studied for its exoenzymes, a chitinase complex (Veldkamp, 1955). The products of the enzymatic activity are *N*-acetylglucosamine, acetate, and ammonia, but not glucose. While chitin decomposition is a common feature of the lysobacters, it appears that so far no other study has been performed on the subject. An extracellular enzyme with both  $\beta$ -1,4-glucanase and chitinase activity was purified from *L. enzymogenes* strain AL-1 (Hedges and Wolfe, 1974). It does not attack chitin. Enzymes of this type are probably responsible for the often-observed hydrolysis of carboxymethyl cellulose. They could also be useful for the study of the structure of fungal cell walls. Strain "Cytophaga" L1 (= NCIB 9497) was patented for its impressive combination of fast-working hydrolytic exoenzymes (Brit. Pat. 1,048,887, 23 November 1966). The enzyme complex includes chitinase, laminarinase, lipase, elastase, kerati-

nase, and other proteolytic activity and may be useful for the decomposition of fungal mycelia from fermentations. Using the same strain, a process was later worked out for the degradation of living yeast cells (Andrews and Asenjo, 1984; Asenjo, 1980; Asenjo and Dunnill, 1981; Asenjo et al., 1981; Hunter and Asenjo, 1987a, 1987b). The yeast lytic enzymes are produced constitutively and consist of  $\beta$ -(1-6)-glucanase, mannanase, and high protease activities. The breakdown of the yeast cells is connected mainly with the protease and the glucanase activities, which have to act in sequence because a specific protease must remove the mannoprotein layer in the outer cell wall before the glucanase can become active. As the glucanase is repressed if more than 0.8 g of glucose is present per liter of medium, a 20-fold-higher enzyme yield is obtained in continuous cultures with optimal dilution rates compared with batch cultures. The lysobacter glucanase appears not to adsorb to the yeast glucan, attacks the fibrillar and the amorphous glucan fractions equally well, and shows no inhibition by its hydrolysis products. If crude enzyme preparations are applied in higher concentrations, the rate of lysis may decline. This may be due to competitive inhibition by carbohydrates present in the enzyme solution. The lysobacter enzyme mixture solubilizes the yeast cells almost completely and thus appears particularly useful for the production of yeast extract and of animal feed. A two-step model has been proposed to describe the kinetics of the process of lysis. Also, the separation of the enzyme complex from the culture broth and its application in an immobilized form has been studied. It appears, however, that the free enzyme is more promising for technical purposes.

Two alkaline phosphatases were demonstrated in *L. enzymogenes* strain 495 (von Tigerstrom, 1983; 1984; von Tigerstrom and Stelmaschuk, 1986, 1987a). The appearance of both activities is repressed by inorganic phosphate. One of those enzymes is excreted into the medium, has a molecular weight of about 25 kDa, and does not contain a metal ion, which is rather unusual for a bacterial phosphatase. As the enzyme is easy to recover and purify, it might be a useful tool in nucleic acid research. The other enzyme appears to be bound to the outer membrane, has a molecular weight of about 69 kDa, and seems to be composed of at least two subunits. It is a metal enzyme probably containing  $Zn^{2+}$ , hydrolyzes a wide variety of 5'-, 3'-, and 2'-ribose and -deoxyribose nucleotides, as well as sugar phosphates, and shows a remarkably high specific activity. A corresponding cell-bound phosphatase was also found in the three other *Lysobacter* species, and all four enzymes are immunologically related among themselves but

completely different from cell-associated phosphatases from other gliding bacteria. The extracellular and the cell-bound phosphatase also differ in their amino acid composition.

The same strain produces two extracellular endonucleases (von Tigerstrom, 1980, 1981). One is a nonspecific nuclease (22–28 kDa) preferentially cleaving double-stranded DNA and, with reduced efficiency, single-stranded DNA and RNA, but not poly(A) and poly(C). It produces large oligonucleotides with 5'-phosphate groups. The synthesis of this enzyme is inhibited by RNA in the medium. The second enzyme is an RNase (46–47 kDa) that also cleaves poly(A) and poly(C) but not double- and single-stranded DNA. It appears to have no base specificity. The enzyme is unusual in that it generates short oligonucleotides with 5'-phosphate ends (instead of the more common 3'-phosphate groups). The production of the RNase is inhibited by phosphate in the medium. During its purification, a contaminating enzyme was discovered that degraded the dialysis bags. The extracellular enzymes of strain 495 are obtained with good yields in tryptone (0.4%) broth at 25°C (von Tigerstrom, 1983). While the production of nuclease, RNase, and phosphatase is inhibited by  $Mg^{2+}$  and by  $Mn^{2+}$  above 0.1 and 0.01 mM, respectively, that of protease is stimulated by  $Mg^{2+}$  and neutral to  $Mn^{2+}$ . The ions appear to interact specifically with the synthesis of the enzymes and not with their release from the cells.

Other hydrolases excreted by lysobacters are: endoamylases found in all four species (von Tigerstrom and Stelmaschuk, 1987b), which seems surprising because the characterization of the lysobacter species excludes starch hydrolysis in almost all strains of three species (see Table 1); the paradox may be a result of the test conditions. The enzyme of *L. brunescens*, the most potent organism, has a molecular weight of 47–49 kDa and also decomposes amylopectin, amylose, and glycogen, but not dextran. The enzyme probably has no commercial interest because of its enzymatic properties and low yield. Further, all species produce two extracellular lipolytic esterases (von Tigerstrom and Stelmaschuk, 1989). One is excreted into the medium and is inducible by olive oil. As it is synthesized only after exponential growth, it appears to be under catabolite repression. The other enzyme is associated with the outer membrane and is constitutive. The two esterases differ in their substrate specificity. Both are very active on Tween 20, but only the free enzyme is able to attack olive oil. In addition, there is a third, cytoplasmic esterase which, in contrast to the extracellular enzymes, is very active on tributyrin. Finally, there is a periplasmic  $\beta$ -lactamase.

The most fascinating *Lysobacter* enzymes are their proteases. Two proteases were isolated from the culture supernatant of *L. enzymogenes* strain (“*Sorangium*” or “myxobacter”) 495; one of them  $\alpha$ -lytic protease, became a very important enzyme for scientific reasons. The strain was originally noted for its ability to rapidly lyse a wide spectrum of bacteria (Gillespie and Cook, 1965) and nematodes (Katznelson et al., 1964). Soon it was realized that the lytic activity was due to at least two different proteases,  $\alpha$ - and  $\beta$ -lytic proteases (Whitaker, 1965), and procedures were worked out to produce and purify those enzymes (Whitaker et al., 1965a; Whitaker, 1967a). Later it was found that there are at least two more proteases in the culture broth.

A major research effort has been made to characterize the  $\alpha$ -lytic protease. This enzyme is an alkaline serine protease (for a summary of the early work see: Whitaker, 1970) that aroused great interest because it was the first bacterial serine protease with the same amino acid sequence in its active center, Gly-Asp-Ser-Gly-Gly, as the mammalian pancreatic serine proteases (Olson et al., 1970; Whitaker et al., 1966; Whitaker and Roy, 1967), and not Thr-Ser-Met as in most other bacterial serine proteases. This suggested a common ancestor for both enzymes and invited studies on the evolution of protein structure and function. The similarity to porcine pancreatic elastase is particularly notable, a similarity which also extends to substrate specificity and kinetic properties (Kaplan and Whitaker, 1969; Kaplan et al., 1970). While the homology in the amino acid sequence is only high in certain critical regions (overall homology 18%), long sections appear conserved in the three-dimensional structure as revealed by high-resolution X-ray analyses and molecular models (55% topological equivalence). There is an even higher correspondence with *Streptomyces griseus* proteases A and B, with 35% and 36% sequence homology and 80% topological equivalence (e.g., Brayer et al., 1979; Fujinaga et al., 1985; McLachlan and Shotton, 1971). The  $\alpha$ -lytic protease (EC 3.4.21.12; 19.8 kDa) consists of 198 amino acids and contains three disulfide bridges. It cleaves polypeptides at the carbonyl side of short neutral aliphatic amino acids, preferentially alanine (Kaplan and Whitaker, 1969). It also attacks bacterial peptidoglycans, mainly at the cross-linkages (Tsai et al., 1965). Like other serine proteases,  $\alpha$ -lytic protease has several subsites (in this case, six) for substrate binding, with different amino acid specificities (Bauer et al., 1981). This explains why the enzyme prefers long substrate molecules over short ones. While the pH optimum for catalytic activity is reached at pH 8, substrate binding is independent of pH

between pH 5 and 10.5 (Kaplan and Whitaker, 1969; Paterson and Whitaker, 1969). The enzyme shows a high stability unparalleled in any other protease (Kaplan et al., 1970), which appears to arise from a high structural rigidity (Fujinaga et al., 1985). As with other serine proteases, a strong H-isotope effect is observed: the catalytic rate drops to one-third in  $^2\text{H}_2\text{O}$  (Hunkapiller et al., 1973). The discovery that  $\alpha$ -lytic protease contains but one histidine (His) residue (Jurášek and Whitaker, 1967) proved particularly momentous for the elucidation of the catalytic mechanism of serine proteases, for the other enzymes all contain more than one His. This finding at once excluded a requirement of several His residues for the catalytic process, as was postulated before. The histidine in  $\alpha$ -lytic protease is homologous with the His-57 in  $\alpha$ -chymotrypsin and is part of the catalytic triad, Asp-102/His-57/Ser-195 (the adopted numbering is that of  $\alpha$ -chymotrypsin). By the use of a His auxotrophic mutant,  $^{13}\text{C}$  and  $^{15}\text{N}$  could substantially be enriched in His-57 (Westler et al., 1982), which allowed refined NMR studies of the dissociation behavior and hydrogen bonding in the active center (Bachovkin, 1986; Bachovkin et al., 1981; Hunkapiller et al., 1973). Another approach was the use of peptide-boronic acid inhibitors forming rather stable enzyme-substrate complexes, which can be analyzed as models of the otherwise-not-accessible transition state (Bone et al., 1987; 1989). Those studies provided deep insights into the mechanism of catalysis and substrate specificity. The gene of  $\alpha$ -lytic protease (the pro gene) has also been cloned in *Escherichia coli* (Epstein and Wensink, 1988; Silen et al., 1988). At the amino end of the protease domain, the gene has an open reading frame that extends 198 (or 199; the two articles do not completely agree in the details) amino acid codons upstream and represents a very large prepro sequence. The latter consists of a typical bacterial pre (or signal) peptide of 24 (or 33) amino acids ending with a Ala-Leu-Ala-Ala cleavage site, followed by a pro region of 174 (or 166) amino acids, i.e., more than twice the size of other known bacterial propeptides. The high sequence homology observed between  $\alpha$ -lytic protease and proteases A and B of *Streptomyces griseus* continues for 49 amino acids into the pro region (21% and 38% homology, respectively), and then falls suddenly to a low value of 7% and 3%. This suggests that the *Lysobacter* pro region is a composite of two parts, perhaps of different origin and with different functions. In fact the amino acid sequence around the cleavage site of the signal peptide shows a unique homology to a trypsin inhibitor of maize, and it was speculated that this part of the peptide may block the protease activity, while the conservative part

may serve an identical function as the (much shorter) propeptides of proteinases A and B, namely, arranging the correct folding of the protease domain (Epstein and Wensink, 1988). The *Lysobacter* ribosomal binding site (AGGAG) is homologous to known *E. coli* binding sites. When the *Lysobacter* gene is fused with an inducible *E. coli* promoter and signal sequence (e.g., pho A),  $\alpha$ -lytic protease is expressed in *E. coli* and (probably unspecifically) exported into the medium (Silen and Agard, 1989; Silen et al., 1989). Deletion of the pro region results in inactive enzyme, which is immunologically and electrophoretically indistinguishable from  $\alpha$ -lytic protease, but presumably improperly folded. Site-specific mutation of the Ser-195 in the catalytic pocket leads to the production of a double-sized inactive protease molecule, which suggests that the removal of the propeptide is an autocatalytic process. Surprisingly, the activation (not the production) of  $\alpha$ -lytic protease proved temperature sensitive and did not take place above 30°C. If the pro region and the protease domain were cloned separately with independently inducible *E. coli* promoters, active enzyme was obtained when both were induced concomitantly, in spite of the fact that in this case, the two peptide were not covalently connected. Obviously, the propeptide functions as a chaperonin-like, though specific, folding template. It appears that  $\alpha$ -lytic protease is produced exclusively by strain 495.

Much less is known about the  $\beta$ -lytic protease, a second protease from strain 495. The enzyme is a nonserine protease and resembles mammalian carboxypeptidase and particularly the bacterial neutral proteases (Oza, 1973). Its specificity is quite distinct from that of the pancreatopeptidases. It cleaves very selectively at the carbonyl side of neutral amino acids. Thus, only the B chain of (denatured) insulin is cleaved, and that between glycine-23 and phenylalanine-24 and, much more slowly, between valine-18 and cysteine-19 (Whitaker et al., 1965b). A free  $\alpha$ -amino group or a free terminal carboxyl next to the cleavage site is not accepted, but an amido group on the carboxyl already eliminates the blocking effect of the latter. The  $\beta$ -enzyme is more active than the  $\alpha$ -enzyme on bacterial peptidoglycan rapidly hydrolyzing the muramic-acid alanine bond (Tsai et al., 1965). The  $\beta$ -lytic protease (19 kDa) appears to be composed of 177 amino acids and to contain two disulfide bridges and one atom of zinc (Jurášek and Whitaker, 1967). The zinc can be removed and restored again with reconstitution of enzyme activity (Whitaker, 1967b). While the apoenzyme is no longer proteolytic (Oza, 1973), the cell-wall-lytic activity was still observed (Whitaker and Roy, 1967). But conceivably the protease was simply restored by

a contamination of the peptidoglycan with zinc. The pH optimum of  $\beta$ -lytic protease is 6.5 (Oza, 1973). The proteases of strain 495 can be produced with good yields (4–4.5 g of  $\alpha$ - and 2 g of  $\beta$ -enzyme from 100 liters) on casamino acid/glucose media (Whitaker, 1967a). Also, production on media with condensed fish solubles, a by-product of the salmon canneries, seems feasible (Wah-On et al., 1980).

Finally, the proteases of *L. enzymogenes* strain ("myxobacter") AL-1 (ATCC 27796) should briefly be mentioned. The organism was isolated because it lysed cells and cell walls of *Arthrobacter crystallopoietes*, and enzymes with that property were of much interest at that time for studies on the cell wall structure of bacteria (Ensign and Wolfe, 1965, 1966). In fact, one of the AL-1 enzymes was soon successfully applied for that purpose (for a review, see Ghuysen, 1968). The enzyme is particularly useful because it cleaves the bond between muramic acid and L-alanine, making it possible to isolate the pure polysaccharide backbones of peptidoglycan. The enzyme could thus be used to demonstrate a shortening of the polysaccharide chain length during the rod-to-sphere conversion of *Arthrobacter* (Krulwich et al., 1967). At first, only the enzyme just mentioned, AL-1 protease or, later, AL-1 protease I, was recognized and characterized (Ensign and Wolfe, 1966; Jackson and Wolfe, 1968; Jackson and Matsueda, 1970). The enzyme can be produced by fermentation in 1% yeast extract medium at 30°C for 36 h, and is recovered from the culture supernatant with a yield of 350 mg of pure enzyme from 100 liters. The AL-1 protease I (13.5 kDa) is an alkaline endopeptidase with a sharp pH optimum at 9.0. The molecule consists of 136 amino acids, with Ser, Asp, Gly, Thr, and Ala accounting for more than 50% of the residues. It contains one disulfide bridge, and does not contain a hexose molecule, as was previously suggested. The enzyme is spherical, compact, and very stable. Within minutes, it completely lyses whole cells of many Gram-positive and some Gram-negative bacteria, as well as purified peptidoglycan (Ensign and Wolfe, 1965). It cleaves about 30% of the bonds in casein and albumin, and 15% in gelatin. In the B chain of insulin only the bonds between Ala and Leu, Gly and Phe, and Val and Cys are attacked. Thus it appears that a hydrophobic residue is required at the cleavage site, but the exact specificity is not yet known. In peptidoglycan, besides the connection of the peptide side chain to the polysaccharide backbone, only the bonds between terminal D-Ala and the pentaglycine or L-Ala bridges, and bonds within the pentaglycine bridge are cleaved. The enzyme therefore became a useful tool for the elucidation of the structure of bacterial cell walls (e.g., Ghuysen, 1968; Jarvis and

Strominger, 1967; Katz and Strominger, 1967; Tipper, 1969).

Also, the second protease from strain AL-1, AL-1 protease II (17 kDa), is a small, stable, alkaline endopeptidase, but it does not attack bacterial cell walls (Wingard et al., 1972). It is obtained from the culture supernatant with a yield of 10% of protease I. The enzyme is very unusual in two respects: it readily crystallizes from the column fractions, and it cleaves exclusively at the amino side of lysine. It does not remove terminal lysine residues. The enzyme consists of 157 amino acids, 50% of which are Asp, Ala, Thr, and Gly, and has a broad pH optimum between 8.5 and 9.0.

A third unusual protease from AL-1 is called endoproteinase Lys-C. This enzyme has a high although not absolute specificity for peptide and ester bonds at the carboxyl end of lysine (information leaflet Boehringer Mannheim, Biochemica-Dienst no. 42, April 1980), so that it can be used for sequencing proteins and for peptide mapping (Au et al., 1989; Boileau et al., 1982; Hofsteenge et al., 1983; Jekel et al., 1983; Jolles et al., 1983). The enzyme (30 kDa unreduced, 33 kDa reduced) is an alkaline serine protease, is very stable even in the presence of 0.1% sodium dodecylsulfate or 5 M urea, and is commercially available from Boehringer Mannheim (Mannheim, Germany).

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## The Genus *Moraxella*

JOHN P. HAYS

The *Moraxella* genus currently contains at least 14 different species, including *M. catarrhalis*, *M. bovis*, *M. lacunata*, *M. osloensis*, *M. nonliquefaciens*, *M. atlantae*, *M. lincolnii*, *M. ovis*, *M. caviae*, *M. canis*, *M. equi*, *M. cuniculi*, *M. caprae* and *M. boeveyi*, which colonize both humans and animals. The genus is under constant revision, with recent taxonomic restructuring placing the bacterial species formerly known as *Moraxella phenylpyruvica* in the genus *Psychrobacter* as *Psychrobacter phenylpyruvica* and *Moraxella urethralis* in the *Oligella* genus as *Oligella urethralis* (Fig. 1).

Common characteristics of the *Moraxella* genus include a lack of colony pigmentation; Gram-negative staining coccobacillus and bacillus morphology (except *M. catarrhalis*, which exhibits a coccoid morphology; Fig. 2), with a tendency to resist decolorization; positive with oxidase reagent and tetra- and dimethyl-*p*-phenylenediamine; and a G+C content of 40–47.5 mol%. Optimum growth conditions are achieved on blood agar plates under aerobic conditions at a temperature of approximately 33–37°C. *Moraxella lacunata* colonies form dark haloes on chocolate agar, most often with pitting of the agar (a phenomenon only occasionally observed for other *Moraxella* spp.). The addition of bile salts and sodium desoxycholate to the growth medium tends to stimulate *M. atlantae* but inhibits the growth of *M. lacunata* and *M. nonliquefaciens*. Combined genetic transformation and nutritional assays for the identification of *Moraxella nonliquefaciens* have been reported (Juni et al., 1984). In general, however, distinguishing between the different *Moraxella* species tends to be difficult (Table 1), not least because of the asaccharolytic nature of the genus, though some publications have indicated that 16S rRNA sequence polymorphisms may be a useful adjunct to biochemical testing (Enright et al., 1994a; Pettersson et al., 1998a; Harmsen et al., 2001). When combined, biochemical and 16S rRNA sequence data do not tend to warrant a distinction of the *Moraxella* genus into two distinct subgenera as suggested by Bovre (1984).

*Moraxella lacunata*, *M. osloensis*, *M. nonliquefaciens*, *M. atlantae* and *M. lincolnii* are commensals of human epithelia that are infrequently associated with human disease. Though only infrequently associated with human disease (*M. catarrhalis* excluded), the range of diseases caused by *Moraxella* spp. in humans is somewhat broad and includes: 1) ocular infections, e.g., conjunctivitis and endophthalmitis; 2) respiratory infections, e.g., “pneumococcal pneumoniae” and chronic bronchitis; and 3) systemic infections, e.g., bacteremia, septic arthritis, acute thyroiditis, neonatal meningitis, central venous catheter infection and bone and joint infection (*panarthritis ossale et articulare*).

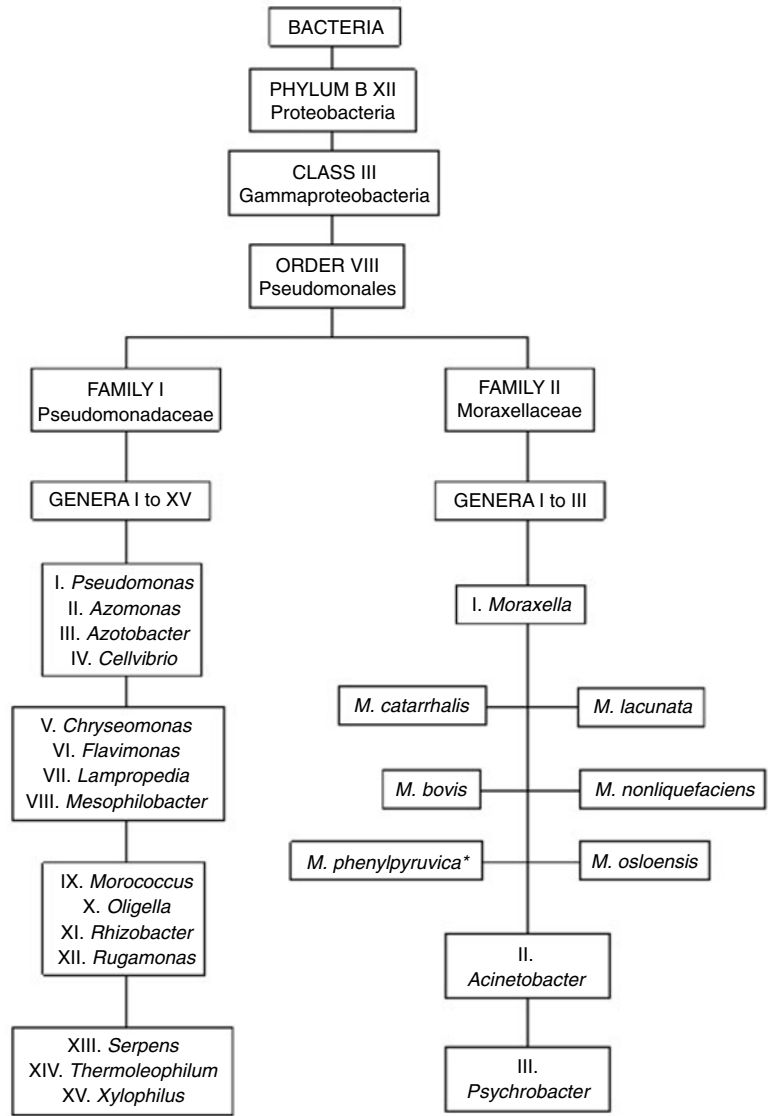
Several *Moraxella* species are associated with animals, including *M. canis* (cats and dogs), *M. ovis* (sheep), *M. equi* (horses), *M. caprae* and *M. boeveyi* (goats), *M. caviae* (guinea pigs) and *M. cuniculi* (rabbits). Like their human counterparts, these animal-associated *Moraxella* spp. are infrequently associated with facilitating disease (*M. bovis* excluded), though *Moraxella* infections related to chronic cholera-like lesions in birds, and keratoconjunctivitis in mule deer and moose, have been described (Emerson, 1983; Dubai, 2000). *Moraxella bovis* is actually an important veterinary pathogen, a situation that mirrors the importance of *M. catarrhalis* as the single most important human pathogen within human commensal *Moraxella* species.

The majority of *Moraxella* spp. is susceptible to penicillin-derived antibiotics, including cephalosporins, tetracyclines, quinolones and aminoglycosides. This contrasts with *M. catarrhalis*, where an alarming increase in the number of penicillin resistant isolates has been observed in the last 30 years (now approximating 90–95% of all isolations). This distinction in antibiotic sensitivity is most probably related to the fact that *M. catarrhalis* produces  $\beta$ -lactamases, a phenomenon apparently rare in other *Moraxella* spp. (though at least one publication has indicated otherwise with respect to *M. nonliquefaciens*; Eliasson et al., 1992).

The exceptional importance of *M. bovis* and *M. catarrhalis* in veterinary and human infection



Fig. 1. The genus *Moraxella* and its positioning in the Order Pseudomonadales, Family Moraxellaceae. (© J. P. Hays.)



\**Psychrobacter phenylpyruvica*

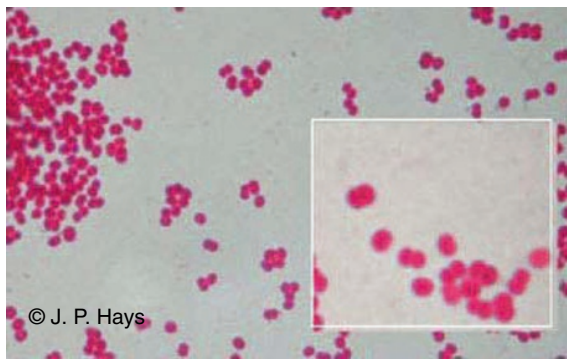


Fig. 2. Gram film of *M. catarrhalis* showing typical Gram-negative diplococcus morphology. (© J. P. Hays.)

within the *Moraxella* genus means that a more detailed discussion of these pathogens is warranted.

***Moraxella bovis***

*Moraxella bovis* is the most common cause of infectious bovine keratoconjunctivitis (IBK), an epidemic and highly contagious disease causing temporary or even permanent blindness, predominantly in calves (Henson and Grumbles, 1960). The bacterium is thought to comprise part of the normal bacterial flora of bovines, colonizing the ocular and nasal epithelia (Moore and

Table 1. Biochemical tests available to distinguish between members of the genus *Moraxella*. (From Murray et al., 1999, with permission.)

Test	<i>Moraxella lacunata</i>	<i>Moraxella nonliquefaciens</i>	<i>Moraxella canis</i>	<i>Moraxella catarrhalis</i>	<i>Moraxella lincolni</i>	<i>Moraxella osloensis</i>	<i>Moraxella atlantae</i>	<i>Psychrobacter phenylpyruvicus</i>	<i>Oligella urethralis</i>	<i>Oligella ureolytica</i>
Motility	-	-	-	-	-	-	-	-	-	+ <sup>c</sup>
Catalase	+	+	+	+	+	+	+	+	+	+
Hemolysis (SBA)	α/-	-	-	-	-	-	-	s1β/-	s1β/-	s1β/-
Growth on MacConkey agar	-	-	+	-	-	V	+	+	+	V
At 42°C	-	V	ND	V	-	-	V	V	+	V
Urease	-	-	-	-	-	-	-	+	-	++
Phenylalanine deaminase	D	-	-	V	ND	V	-	+	+	+
Gelatin hydrolysis	+ <sup>c</sup>	-	-	-	-	-	-	-	-	-
Nitrate to nitrite	+	+	+	+	-	V	-	V	-	+
Nitrite reduction	-	-	V	+	V	-	-	-	+	+
DNase	-	-	+	-	-	-	-	-	-	-
Penicillin <sup>d</sup>	S	S	S	R/S <sup>e</sup>	S	S	S	V	S	S
CFA <sub>s</sub>	16:0, 16:1ω7c, 18:1ω9c	18:1ω9c, 16:1ω7c, 16:0	18:1ω9c	16:1ω7c, 17:1ω8c, 18:1ω9c	16:0, 16:1ω7c, 18:1ω9c	16:1ω7c, 18:1ω9c	16:0, 18:2, 18:1ω9c, 18:0	16:0, 16:1ω7c, 18:1ω9c, 18:2	16:0, 18:1ω7c	16:0, 18:1ω7c

Symbols and abbreviations: +, positive; -, negative; ++, strong positive reaction; CFA<sub>s</sub>, *cis* fatty acids; SBA, sheep blood agar; α, alpha-hemolysis; s1β, diffuse β-hemolysis underneath colonies on SBA; D, different data reported in literature; S, susceptible; and R, resistant.

<sup>a</sup>For truest microscopic morphology, perform Gram staining with a sample from enrichment broth culture incubated for 2 h at 35°C.

<sup>b</sup>*Brucella* species may be misidentified as *Psychrobacter (Moraxella) phenylpyruvicus* in some commercial identification systems.

<sup>c</sup>May be difficult to demonstrate.

<sup>d</sup>Test performed by directly inoculating surface of SBA in a manner that will produce a uniform lawn of growth, immediately placing a 10-U penicillin disc on the agar surface, and incubating in room atmosphere at 35°C for 18–24 h. Any strain with a zone of inhibited growth around the penicillin disc is interpreted as susceptible for identification purposes.

<sup>e</sup>Some strains, particularly those in Europe, may be β-lactamase negative and therefore penicillin susceptible.

Rutter, 1989; Kodjo et al., 1993) with spread of the organism within herds possibly occurring via face flies (Diptera: Muscidae; Arends et al., 1984; Glass and Gerhardt, 1984). Some evidence suggests that not all *M. bovis* infections result in overt disease (Powe et al., 1992).

### Isolation and Identification

Several selective methods for the isolation of *M. bovis* from animal derived specimens have been described, including the use of Tween 80 agar (Mattinson and Cox, 1982), and medium MB (but not medium Mn-B; Juni et al., 1987; Juni et al., 1988), as well as growth on conventional media (e.g., blood agar, GC agar base with 1% IsoVitaleX; Pugh et al., 1966). Further, Webber (1982) indicated that the incorporation of 2.5 micrograms/ml of cloxacillin into 5% bovine blood agar may provide an inexpensive culture medium for the primary isolation of *M. bovis* from bovine lacrimal and nasal secretions (Webber et al., 1982). Both antibody techniques (Nayar and Saunders, 1973; Turfrey, 1985; Wanemuehler et al., 1989) and molecular methods (e.g., multilocus enzyme electrophoresis and hybridization with pilin-specific DNA probes) have also been used to distinguish *M. bovis* from other *Moraxella* spp. (Tonjum et al., 1992).

Interstrain variability and typing of *M. bovis* isolates has been performed using a series of techniques, including 1) serology (slide agglutination [SA], enzyme-linked immunosorbent assay [ELISA], and tandem-crossed immunoelectrophoresis [TCIE]; Gil-Turnes and de Araujo, 1982; Gil-Turnes and Albuquerque, 1984; Moore and Rutter, 1987; Moore and Lepper, 1991); 2) a combination of genetic, outer membrane protein (OMP) and lipopolysaccharide (LPS) analyses (Prieto et al., 1999); and 3) random amplification of polymorphic DNA (RAPD; Conceicao et al., 2004). Plasmid identification has also been suggested as an additional useful method for *M. bovis* strain identification since a range of plasmids (up to 45 kb in size) has been identified within the species (McDonald and Pugh, 1986; Wilt et al., 1989).

### Pathogenicity

*Moraxella bovis* exhibits several pathogenic traits, including the expression of adhesins, leukotoxin, phosphoamidase, phosphatase, hyaluronidase, lipases, etc. (Frank and Gerber, 1981; Gil-Turnes and Aleixo, 1991). However, of particular importance with respect to virulence, is the presence or absence of pili (fimbriae; Jayappa and Lehr, 1986; Lepper and Power, 1988b; Ruehl et al., 1988) and the expression of

a hemolysin or cytolysin (Kagonyera et al., 1989).

Pili (fimbriae) have been shown to mediate adherence of *M. bovis* to bovine corneal cells in vitro (Annuar and Wilcox, 1985), and nonpiliated strains are nonvirulent during experimental corneal infection of laboratory mice and cattle (Jayappa and Lehr, 1986). Interestingly, single isolates possess the capacity to make more than one type of pilin (Marrs et al., 1988). For example, strain Epp 63 (the most intensively studied) expresses alpha (I) and beta (Q) fimbrial variants, which possess approximately 70% sequence identity. Moreover, the beta (Q) variant is significantly more virulent than the alpha (I) or nonpiliated variants (Ruehl et al., 1988; Ruehl et al., 1993). Further, phase variable expression of the pilin genes per se (i.e., piliated versus nonpiliated and vice versa) has also been demonstrated (Bovre and Froholm, 1972).

Hemolysin is a calcium-dependent, repeats-in-toxin (RTX)-related toxin of 94–110 kDa molecular mass (Gray et al., 1995; Billson et al., 2000), which is secreted by most pathogenic *M. bovis* cells. The hemolysin creates cellular pores that ultimately inflict damage to bovine erythrocytes (e.g., by making them swell and leak internal solutes; Clinkenbeard and Thiessen, 1991) as well as bovine neutrophils (facilitating cytotoxicity; Hoiem-Dalen et al., 1990). The *M. bovis* hemolysin per se is encoded by the *mbxA* gene, part of a typical RTX operon comprising four genes arranged *mbxCABD* (5'-3'). Flanking this operon lies putative transposases (5') and *tolC*-like genes (3'), with nonhemolytic strains apparently lacking the operon (Angelos et al., 2003). The hemolysin may be inactivated by heat, trypsin, formalin, or lyophilization (Sandhu and White, 1977; Ostle and Rosenbusch, 1984), and in broth cultures, reaches a maximum concentration approximately 4.5 h after inoculation (late logarithmic phase). Cattle with a history of infectious bovine keratoconjunctivitis are found to have high antihemolysin antibody titers in their blood (Ostle and Rosenbusch, 1985), and vaccination with hemolytic cell-free extracts has been shown to protect against IBK (Billson et al., 1994).

### Treatment and Vaccination

The use of antibiotics is a viable option in treating *M. bovis*-mediated keratoconjunctivitis (Pugh and McDonald, 1977a; George et al., 1984), with most antibiotics showing low minimum inhibitory concentrations (MICs) against *M. bovis* including ampicillin, ceftiofur, tilmicosin, tylosin, erythromycin, oxytetracycline and gentamicin (Shryock et al., 1998). In particular, long-acting oxytetracycline has been proven to

reduce corneal ulceration (Smith and George, 1985) and lower the incidence of keratoconjunctivitis (George and Smith, 1985). However, differences in antibiotic sensitivity between individual isolates have been determined (Gil-Turnes and Albuquerque, 1984).

Vaccination is also under investigation as a means of controlling *M. bovis* infection. However, different *M. bovis* isolates produce serologically different types of pili (Sandhu et al., 1974), and a single isolate may express different pili forms at different times, which means that protective immunity is generally only conferred by antiserum raised against homologous isolates (Pugh et al., 1977b; Lepper, 1988a, Moore and Rutter, 1989). Moreover, there are indications that switching of pilin type within individual isolates (to a different pilus serotype) may occur after vaccination with a single homologous pilus serotype (Lepper et al., 1995). The use of a polyvalent subunit pilus vaccine in protecting cattle against IBK has been proposed and used with varying degrees of success (Lepper et al., 1992; Lepper et al., 1995). Other *M. bovis* vaccines have used pooled bacterin from various non-hemolytic strains, or ribosomes, and have had mixed success in preventing IBK in calves (Pugh et al., 1981; Pugh et al., 1982). The use of adjuvants (e.g., diphtheria-tetanus-toxoids and pertussis vaccine [DPT]) has been shown to be useful in promoting the development of a serological response to pili-related vaccines (Pugh et al., 1984).

### *Moraxella catarrhalis*

The bacterial species known as *Moraxella catarrhalis* (also referred to as "*Branhamella catarrhalis*" or "*Moraxella [Branhamella] catarrhalis*") was originally thought to be a common human commensal bacterium with little pathogenic potential, a characteristic currently shared by most other species within the *Moraxella* genus. However, evidence collected over the last 30 years has proven the clinical relevance of this organism in facilitating disease in both adults and children. The main question now remaining is the bacterila/host mechanism(s) allowing the organism to switch from being a harmless commensal to a facilitator of pathogenic disease.

#### Taxonomy

*Moraxella catarrhalis* has undergone several name changes since its first discovery in 1896 (Frosch and Kolle, 1896). Originally entitled *Micrococcus catarrhalis*, the organism became more familiar as *Neisseria catarrhalis* in the 1950s, largely due to its Gram-negative, diplococoid morphology and rapid oxidase positive

reaction (characteristics associated with *Neisseria* species). In 1963 however, Berger showed that this genus may actually contain two distinct species (*Neisseria cinerea* and *Neisseria catarrhalis*), which could be separated by their biochemical ability to reduce nitrate and nitrite and to utilize tributyrin (Berger, 1963). With the introduction of DNA technology, further phylogenetic studies indicated a lack of chromosomal DNA homology, between the "true" *Neisseria* spp. and *N. catarrhalis*, so much so, that in 1970 the species was renamed "*Branhamella catarrhalis*" and relocated to the new genus of *Branhamella* to honor the famous microbiologist Sarah E. Branham who had previously conducted research on *Neisseria* (Catlin, 1970). Fourteen years later, Bovre suggested that *B. catarrhalis* be renamed "*Moraxella (Branhamella) catarrhalis*" and be re-assigned to the genus *Moraxella*, not least because of the distinct physiological and genetic relatedness between *Branhamella catarrhalis* and members of the *Moraxella* genus (Bovre, 1984). In 1991, Catlin suggested the formation of a new family (the Branhamaceae), which would accommodate both *Branhamella* and *Moraxella* genera and take into account the differences in morphology (cocci versus rods) and pathogenicity (important mucosal pathogens versus rare causes of disease) (Catlin, 1991). However, this suggestion has not been adopted by taxonomists, not least because 16S ribosomal RNA sequence analysis and genetic hybridization studies tend to favor the current classification of *M. catarrhalis* (Enright et al., 1994b; Pettersson et al., 1998b). Currently, the *Moraxella* genus contains both rod-shaped and coccoid bacteria exhibiting similar genetic relatedness, though taxonomic revision of the genus is a continuing process. Indeed, growing evidence suggests that the current *M. catarrhalis* genus may actually represent two different subspecies or even distinct species per se (Bootsma et al., 2000a; Verduin et al., 2000).

#### Isolation and Identification

Optimum growth conditions for *M. catarrhalis* include a temperature of 35–37°C in an atmosphere containing 3–7% CO<sub>2</sub>, though the organism is also capable of growing at a larger range of temperatures (20–42°C) and in ambient air. Pure cultures of *M. catarrhalis* may be grown on standard laboratory media including Mueller Hinton agar, Columbia blood agar, and brain heart infusion or tryptic soy digest broths, though the addition of supplements, e.g., boiled blood plus vitamin and amino acid enrichment (chocolate agar), may result in an enhancement of growth (Doern and Morse, 1980). *Moraxella catarrhalis* does not grow on Modified Thayer-

Martin (MTM) medium. A defined medium for *M. catarrhalis* culture has been described by Juni et al. (Juni et al., 1986), however the growth of *M. catarrhalis* from clinical specimens may be complicated by the presence of the normal bacterial flora, and in particular, the presence of nonpathogenic *Neisseria*. For this reason, selective agars may best be used for the isolation of *M. catarrhalis* from clinical specimens such as sputum etc. Several types of selective media have been described for *M. catarrhalis* with a common feature being the inclusion of trimethoprim, vancomycin, and an antifungal agent, with the further addition of acetazolamide (a synthetic sulfonamide) having been reported to enhance recovery (Vanechoutte et al., 1988). Of interest, *M. catarrhalis* isolated on modified in New York City (MNYC) medium containing antibiotics selective for pathogenic *Neisseria* may possess a greater virulence potential than isolates that do not grow on this medium (El-Ahmer et al., 2003). For long-term storage (several years) at  $-80^{\circ}\text{C}$ , STGG (skimmed milk, tryptone, glucose, glycerol) medium has been reported to yield encouraging results (Kaijalainen et al., 2004).

*Moraxella catarrhalis* colonies tend to be large, gray or nonpigmented, smooth, opaque and convex in nature and may be readily pushed intact over the surface of agar using a sterile loop. In a typical Gram film, the organism appears as a Gram-negative diplococcus with flattened sides, though physical appearance by itself is not enough to separate the species from related contaminating *Neisseria* spp. The identification of *M. catarrhalis* to the species level involves biochemical testing with the production of oxidase, expression of a DNase, lack of acid production from glucose, sucrose, lactose, maltose and fructose, reduction of nitrate and nitrite, and the hydrolysis of tributyrin being important, though none of the tests by themselves are 100% specific or sensitive (Catlin, 1990). The use of DNA technology, and in particular the polymerase chain reaction (PCR) technique, has allowed the direct detection of *M. catarrhalis* from clinical isolates (middle ear effusions, nasopharyngeal secretions etc.) without the prior need for culture. The validity of PCR detection has been proven in animal models (Post et al., 1996; Aul et al., 1998). Indeed, results show a superior sensitivity for PCR (down to six or seven genome equivalents, over more traditional culturing methods; Post et al., 1996). Recent research has also shown that PCR may be used to follow quantitative changes in *M. catarrhalis* numbers in nasopharyngeal secretions in “real-time,” perhaps offering a sensitive and reliable means of following disease progression and treatment regimens in the future (Greiner et al., 2003). However, perhaps the most useful aspect of PCR, with regard to clinical

diagnosis, is the ability of multiplex PCR protocols to detect several pathogens in a single reaction mix, including the acute otitis media (AOM) bacterial pathogens *M. catarrhalis*, *Hemophilus influenzae* and *Streptococcus pneumoniae*. Such multiplex PCRs have already been validated in animal models (Bakaletz et al., 1998) and have been shown to be a reliable diagnostic tool for several screening of middle ear effusions for all pathogens (including *Alloicoccus otitidis*) within a single reaction tube and within a single working day (Hendolin et al., 1999).

### Bacterial Typing and Epidemiology

Traditionally, typing of *M. catarrhalis* isolates (for determining the relatedness of isolates during nosocomial outbreaks etc.), has been performed using a series of nonmolecular techniques, including susceptibility to human serum mediated bacteriolysis, trypsin sensitivity and agglutination profiles against human group O erythrocytes (Soto-Hernandez et al., 1989), isoelectric focusing of  $\beta$ -lactamase proteins (Nash et al., 1991), serological typing of lipopolysaccharide (Van Hare et al., 1987; Vanechoutte et al., 1990), electrophoretic profiling of outer membrane proteins (Bartos and Murphy, 1988; Murphy, 1989a), autoaggregation properties (Janicka et al., 2002), as well as a few other miscellaneous techniques (Denamur et al., 1991; Peiris and Heald, 1992; Peiris et al., 1994). However, advances in DNA technology have allowed comparative whole genomic testing to be performed. The simplest of these techniques involves the use of restriction endonuclease enzymes to specifically digest the genomic DNA of isolates, followed by gel electrophoresis to separate the individual fragments (Patterson et al., 1989; Kawakami et al., 1994; Christensen et al., 1995). *HaeIII*, *HindIII*, *PstI*, *ClaI* and *SpeI* are particularly useful enzymes in this respect. Particularly successful and popular at present is the use of macrorestriction analysis (pulsed field gel electrophoresis or PFGE), which has been used to type *M. catarrhalis* isolates, particularly in the nosocomial situation (Martinez et al., 1999; Vu-Thien et al., 1999; Yano et al., 2000; Hays et al., 2003b; Masaki et al., 2003; Fig. 3). Interestingly, the use of several genomic techniques, including strain-specific DNA probes (Beaulieu et al., 1991; Walker et al., 1998), automated ribotyping, and amplification fragment length polymorphism (AFLP) analysis (Bootsma et al., 2000a; Verduin et al., 2000) have all indicated the division of *M. catarrhalis* isolates into two distinct phylogenetic clusters (possibly subspecies). Further, *M. catarrhalis* isolates expressing various virulence traits (complement resistance and increased adherence to epithelial cells) have

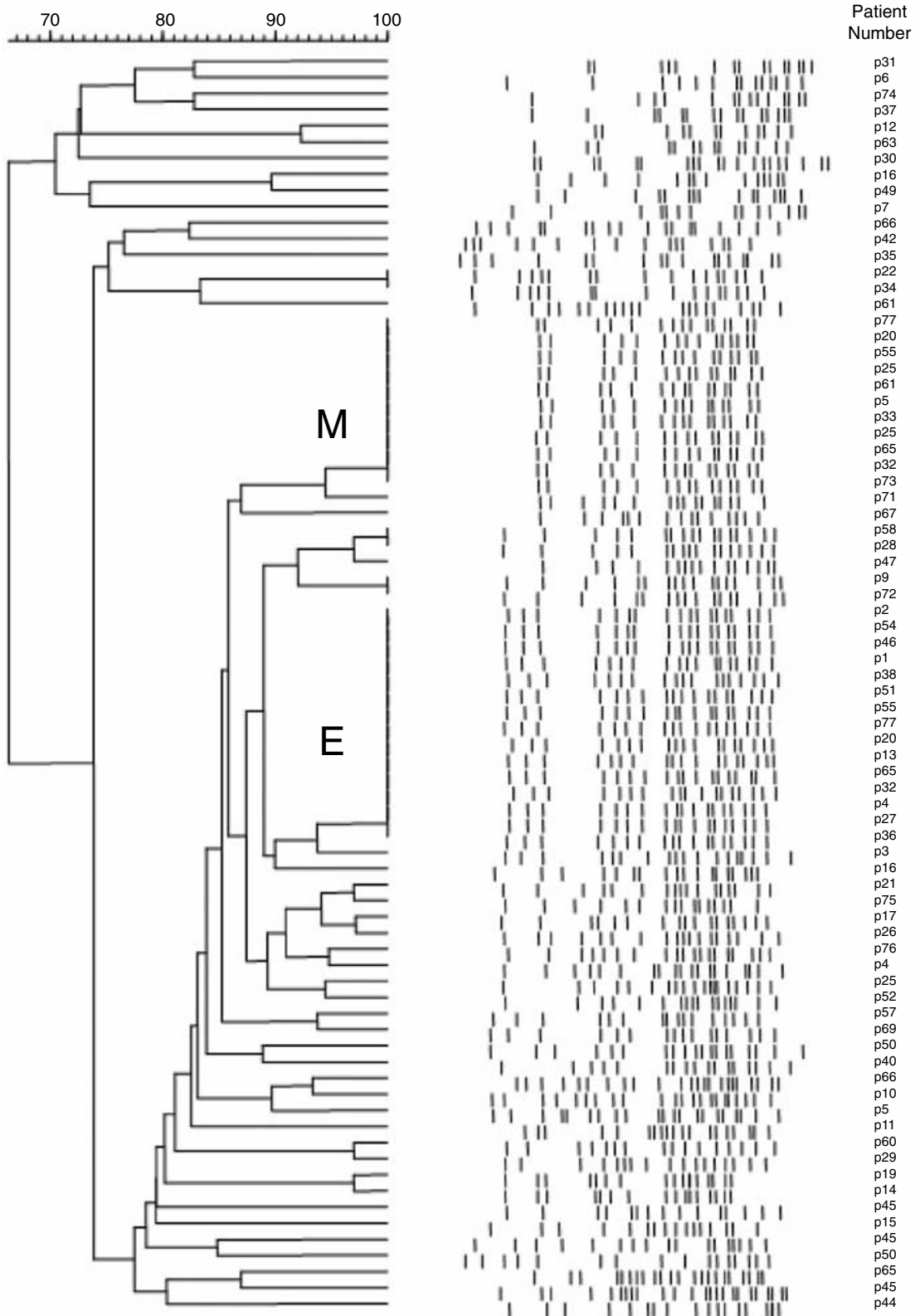


Fig. 3. Dendrogram showing pulsed field gel electrophoresis (PFGE) patterns obtained from *M. catarrhalis* isolates isolated from children admitted to or attending a pediatric intensive care unit in the Netherlands. Letters E and M represent isolates with similar PFGE patterns. (From Hays et al., 2003a, with permission.)

been found to be statistically associated with one of these two clusters (Bootsma et al., 2000a; Verduin et al., 2000). Such genomic typing studies have also shown the genetically diverse nature of *M. catarrhalis* isolates, with successive clonal expansions (often linked to geographic location) apparently occurring (Enright and McKenzie, 1997; Martinez et al., 1999). Interspecies horizontal transfer of genes between *M. catarrhalis* and other species that inhabit the same niche also appears possible (Bootsma et al., 2000b; Luna et al., 2000).

### Associated Diseases

Although once considered a harmless commensal, *M. catarrhalis* is now recognized as an important pathogen of humans and associated with upper and lower respiratory tract infections in both children and adults (Karalus and Campagnari, 2000). In particular, *M. catarrhalis* tends to be associated with upper respiratory tract infections (including acute otitis media and sinusitis) in children, but lower respiratory tract infections in adults (usually with pre-existing pulmonary disease). Occasionally, the bacterium is reported as the causative agent of certain nonrespiratory tract associated infections, including bacteremia (Doern et al., 1981; Domingo et al., 1995; Ioannidis et al., 1995; Abuhammour et al., 1999), endocarditis (Neumayer et al., 1999; Stefanou et al., 2000), conjunctivitis and keratitis (Macasai et al., 1988; Abbott, 1992a; Weiss et al., 1993), and tracheitis (Ernst and Philp, 1987). Less frequently, the organism has been associated with meningitis (Daoud et al., 1996), ventriculitis (Cooke et al., 1990; Rotta et al., 1995), ophthalmia neonatorum (Paul et al., 2000), acute mastoiditis (Leskinen and Jero, 2003), and septic arthritis (Abbott and Nageswaran, 1992b; Olivieri et al., 2004). Interestingly, many reports have indicated the seasonal incidence of *M. catarrhalis* infection, with the winter and spring months showing the greatest rates of isolation (Pollard et al., 1986; Sarubbi et al., 1990; Wood et al., 1996). Moreover, this seasonality is not a prominent feature of respiratory infection caused by other associated bacteria, e.g., *S. pneumoniae* or *H. influenzae* (DiGiovanni et al., 1987; Davies and Maesen, 1988; Kilpi et al., 2001), and there is some speculation that seasonal viral respiratory tract infection (e.g., respiratory syncytial virus [Allgood and Kenny, 1989; El-Ahmer et al., 2003] but not adenoviruses [Bakaletz et al., 1995]) may be a predisposing factor in the seasonality of *M. catarrhalis* infection. Another theory involves bacterial-bacterial competition involving *Corynebacterium* spp., which form part of the normal human throat microflora (Rikitomi et al., 1989).

From a clinicians perspective, it is also important to note that *M. catarrhalis* may be isolated as part of a mixed population of pathogens. For example, research has indicated that isolation of *M. catarrhalis* from sputa is accompanied by the isolation of other bacterial pathogens on approximately 40–50% of occasions (the most common being *S. pneumoniae* and *H. influenzae*; Nicotra et al., 1986; Pollard et al., 1986; Hager et al., 1987). In such cases, defining the role of *M. catarrhalis* in the disease process may be clinically important, particularly with respect to antibiotic therapy, i.e. should antibiotic therapy be targeted against the co-isolated organism only, *M. catarrhalis* only, or should a broad spectrum antibacterial agent be used?

### CHILDHOOD INFECTIONS

*Otitis media* Acute otitis media (AOM) is the most frequent infection associated with *M. catarrhalis* in children (Faden et al., 1990; Faden et al., 1991; Faden et al., 1997; Faden, 2001) and comprises inflammation of the middle ear accompanied by a liquid effusion (Klein et al., 1989; McCormick, 2002). Approximately 50% of children will have experienced at least one episode of AOM by their first birthday, with this proportion rising to 70% by 3 years of age (Teele et al., 1989a; Teele et al., 1989b; Klein, 1994; Turner et al., 2002), representing a tremendous disease burden for this age group and necessitating the widespread use of antibiotics (Cohen et al., 2000; Pichichero, 2000; Pichichero and Casey, 2002). *Moraxella catarrhalis* has been recognized as a specific pathogen associated with AOM for over 70 years (Hart, 1927), and is the third most common bacterium isolated from this condition (behind *H. influenzae* and *S. pneumoniae*; Comisso et al., 2000; Gehanno et al., 2001; Patel et al., 2002). During routine visits, Faden et al. (1994a) observed that otitis media prone children were colonized on 44.4% of occasions compared to only 16.7% of occasions for otitis non-prone children, suggesting a link between increased *M. catarrhalis* colonization and the occurrence of otitis media. However, a genetic relationship between colonizing *M. catarrhalis* isolates and otitis media was not found (Hays et al., 2003b). Of note, the actual rate of *M. catarrhalis* associated AOM may be underestimated, as tympanocentesis and culturing of middle ear effusions is not routinely performed. Also, the development and use of vaccines against *H. influenzae* and *S. pneumoniae* could facilitate an increase in the percentage of AOM episodes attributed to *M. catarrhalis*. The use of new PCR-based methodologies also provides evidence for the presence of *M. catarrhalis* in culture negative middle ear effusions from children suffering

chronic otitis media (Post et al., 1996). Since 1980, the number of reported *M. catarrhalis* isolations from middle ear effusions of children suffering from AOM appears to have markedly increased (now approximating 15–20%), an increase also accompanied by the appearance of  $\beta$ -lactamase producing strains which now account for approximately 90–95% of all isolates (Kovatch et al., 1983; Marchant et al., 1992; Patel et al., 1995; Block, 1997). There is some suggestion that *M. catarrhalis* may facilitate the spread of  $\beta$ -lactamase genes to other bacterial species during mixed bacterial infections (Kadry et al., 2003). It is perhaps poor consolation that the actual numbers of bacteria isolated from middle ear effusions and the severity of symptoms during AOM episodes appear to be lower when *M. catarrhalis* is isolated rather than *S. pneumoniae* or *H. influenzae* (Faden et al., 1992a). Recent recommendations for the treatment of acute otitis media have been presented by Hoberman et al. (2002).

**Sinusitis** Sinusitis is another very common infection in childhood, having been estimated to account for approximately 5–10% of upper respiratory tract infections within this age group (Wald et al., 1989; Wald, 1992a; Wald, 1992b; Wald, 1997; Wald, 1998). Accurate diagnosis may be difficult due to the presence of fairly nonspecific symptoms, which are often similar to allergy or upper respiratory tract viral disease (Daele, 1997). Clinically, sinusitis may be divided into two separate groups, the acute group (where symptoms persist for 10–30 days) and the subacute group (where symptoms persist for 30–120 days). In both cases, *M. catarrhalis* accounts for approximately 5–10% of bacterial infections, with *S. pneumoniae* and *H. influenzae* being more commonly isolated (Chan and Hadley, 2001; Brook, 2002; Finegold et al., 2002). There is some suggestion however, that the rate of isolation of *M. catarrhalis* may be underestimated with respect to sinusitis, as the bacterium grows poorly in environments containing reduced oxygen concentrations (Brorson et al., 1976).

#### *Lower Respiratory Tract Infections in Children*

The role of *M. catarrhalis* in the etiology of lower respiratory tract infections (LRTI) in children seems to be clear-cut. The organism has been isolated in pure culture from tracheal aspirates obtained from neonates, infants and children with pneumoniae (Haddad et al., 1986; Berg and Bartley, 1987; Boyle et al., 1991; Berner et al., 1996). Interestingly, *M. catarrhalis* appears to be increasingly isolated from LRTI originating in hospitalized children, as Chong et al. (1997) indicated an increase in LRTIs in hospitalized chil-

dren from 11.4% (in 1988) to 34.7% (in 1995), a result possibly due to the increasing penicillin resistance observed in *M. catarrhalis* isolates over this period.

#### ADULT INFECTIONS

**Bronchitis and Pneumonia** Though not a common cause of lower respiratory tract infection in healthy adults, *M. catarrhalis* may present a particular problem for adults with pre-existing pulmonary disease and in the elderly. In particular, bacterial exacerbations of chronic obstructive pulmonary disease (COPD) are often attributable to *S. pneumoniae*, nontypeable *H. influenzae*, and *M. catarrhalis* infection in that order (McLeod et al., 1986; Nicotra et al., 1986; Pollard et al., 1986; Boyle et al., 1991; Eller et al., 1998; Miravittles et al., 1999; Anthonisen, 2002; Sethi et al., 2002).

Bacterial pneumonias attributable to *M. catarrhalis* tend to be relatively mild, with the presence of patchy noncavitary infiltrates on chest X-ray (Wright and Wallace, 1989). Particularly susceptible to *M. catarrhalis* pneumonia are patients with end-stage pulmonary or malignant disease (Hager et al., 1987; Barreiro et al., 1992), and many of these (>70%) are, or have been, smokers (DiGiovanni et al., 1987). *Moraxella catarrhalis* pneumonia may occasionally be complicated by bacteremia (Collazos et al., 1992; Ioannidis et al., 1995; Thorsson et al., 1998).

Klingman et al. (1995) indicated that a proportion (approximately 20%) of *M. catarrhalis*-mediated bronchiectasis patients may be chronically colonized with up to four different strains of *M. catarrhalis*, though a direct relationship between the exacerbation of bronchiectasis and *M. catarrhalis* isolation was not proven.

**Laryngitis** *Moraxella catarrhalis* appears to be the most frequent bacterial pathogen associated with laryngitis in adults (Schalen, 1988; Schalen et al., 1992; Schalen et al., 1993), though some debate remains as to whether the organism actually infects or simply colonizes adults presenting with laryngitis (Hol et al., 1996). Further, the pathogenic potential of *M. catarrhalis* in laryngitic disease has been suggested by the fact that laryngitis has been induced in an animal model (Jecker et al., 1999) and patients with laryngitis are more likely to harbor isolates presenting with a complement-resistant phenotype (Hol et al., 1996).

#### Carriage and Spread

*Moraxella catarrhalis* is an organism able to colonize the human respiratory tract without causing disease, though a striking difference exists



between the carriage rates of *M. catarrhalis* in children and in adults. In children, the carriage rate may be as high as 75% (Van Hare et al., 1987; Faden et al., 1994a; Varon et al., 2000; Garcia-Rodriguez and Fresnadillo Martinez, 2002), while in adults the carriage rate is very low at around 1–3% (Denamur et al., 1991; Ejlertsen et al., 1994a). This inverse relationship between rate of colonization and age has been known for approximately 100 years (Arkwright, 1907) and is still present today, though an adequate explanation for this phenomenon still remains elusive. On the basis of the evidence provided by Meier (2003) and Stutzmann Meier (2003), one possible explanation for this phenomenon is that colonization is dependent upon the development of strain-specific mucosal immunoglobulin A (IgA), with specific IgA production perhaps being more important than specific IgG production (Ejlertsen et al., 1994b). In this scenario, multiple colonization and infection with different isolates of *M. catarrhalis* gradually increases the repertoire of specific neutralizing IgA (or IgG) antibodies, such that the number of novel *M. catarrhalis* isolates able to colonize a particular individual decreases with time. Alternatively, otitis prone children may fail to produce a broadly protective antibody response (Faden, 2001) or could possibly have (subclinical) deficiencies in immune modulatory genes. Whatever the explanation, the frequency of colonization appears to be distinctly correlated with the development of disease in children (Brorson and Malmvall, 1981; Van Hare et al., 1987; Faden et al., 1990; Faden et al., 1994a; Prellner et al.,

1994), a correlation that may also hold true for adults (Klingman et al., 1995; Murphy, 1998).

The mechanism of spread of *M. catarrhalis* is still debatable, with possible mechanisms including person-to-person transmission or spread via environmental contamination (Calder et al., 1986; McLeod et al., 1986; Ikram et al., 1993; Hays et al., 2003a). Both nursery schools and day care centers may be important reservoirs for strain exchange in children (Prellner et al., 1984; Yano et al., 2000; Masuda et al., 2002), and intrafamilial spread has been observed (Faden et al., 1994a). Outbreaks of nosocomial infection due to *M. catarrhalis* have been regularly documented (Haddad et al., 1986; Patterson et al., 1988; Berk and Verghese, 1989; Cook et al., 1989; Denamur et al., 1989; Richards et al., 1993; Hays et al., 2003a; Masaki et al., 2003; Fig. 4). However, the degree of (nosocomial) spread may be somewhat underestimated because *M. catarrhalis* colonization does not always appear to lead to the appearance of overt disease (Ikram et al., 1993; Hays et al., 2003a).

### Antibiotic Susceptibility

*Moraxella catarrhalis* exhibits an almost universal resistance to penicillin-related antibiotics, with several recent studies indicating that, worldwide, 90–100% of *M. catarrhalis* isolates produce  $\beta$ -lactamase (Jorgensen et al., 1990; Wallace et al., 1990; Manninen et al., 1997; Zhanel et al., 2000; Abe et al., 2002; Memish et al., 2002; Jones et al., 2003). This is a striking statistic when one considers that before 1970 few isolates produced

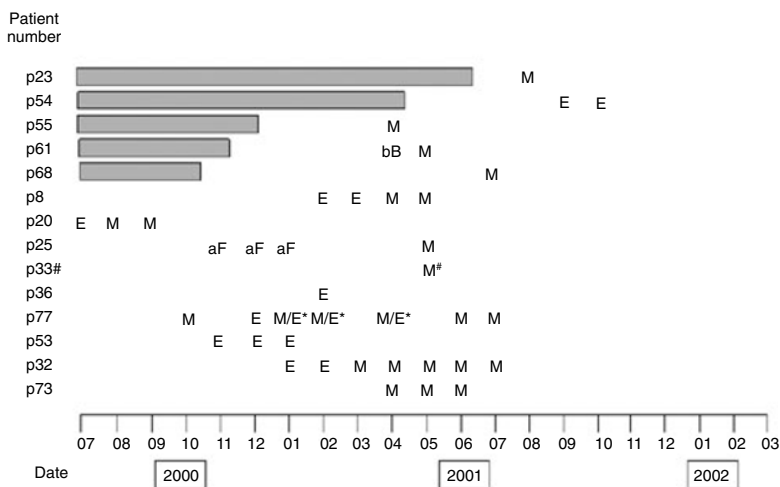


Fig. 4. Timeline showing dynamic population changes between *M. catarrhalis* pulsed field gel electrophoresis (PFGE) types E and M in a pediatric intensive care unit in the Netherlands (see also Fig. 3). Filled rectangles = child not yet born. Letters = pulsed field electrophoresis type assigned. p23 = child number 23. \* = both PFGE types E and M were isolated during these months. # = isolate cultured from a child who had previously been admitted to pediatric intensive care, but at the time of *M. catarrhalis* culture, was present on an unrelated child health ward (none of the other children had been admitted to this particular child health ward). (From Hays et al., 2003a, with permission.)

$\beta$ -lactamase enzymes (Wallace et al., 1989; Catlin, 1990). In fact, the increase in  $\beta$ -lactamase producing isolates has been so rapid that in most countries, a "saturation point" appears to be being reached, with the increase in incidence of  $\beta$ -lactamases positive *M. catarrhalis* strains now leveling off (at this very high level; Shurin et al., 1983; Zhanel et al., 2000; Jones et al., 2002). Research into *M. catarrhalis*  $\beta$ -lactamase production has shown that three different isotype groups may be identified, BRO-1, BRO-2 and BRO-3 (Eliasson and Kamme, 1985; Labia et al., 1986; Christensen et al., 1991). However, by far the most common types are BRO-1 and BRO-2, which are found in approximately 94% and 5% of  $\beta$ -lactamase producers, respectively (Chaibi et al., 1995). There is some suggestion that BRO-1 is gradually replacing BRO-2 over time (Bootsma et al., 2000b; Richter et al., 2000; Ewy and Walker, 2004) and that BRO-1 producing strains are less susceptible to penicillins than BRO-2 producing isolates (Ikeda et al., 1993). Both BRO-1 and BRO-2 enzymes appear to be constitutively expressed, chromosome- or plasmid-encoded proteins, which differ by a single amino acid (Eliasson and Kamme, 1985; Kamme et al., 1986; Eliasson et al., 1992; Bootsma et al., 1996). Interestingly, some evidence suggests that the BRO  $\beta$ -lactamases of *M. catarrhalis* were originally derived from a non-*Moraxella* species; this evidence includes the lower G+C content of the BRO-encoding (*bla*) genes (31% compared to an average of 41%), and the fact that the BRO-type  $\beta$ -lactamases are lipidated in *M. catarrhalis* (suggesting a Gram-positive origin for the gene; Wallace et al., 1989; Bootsma et al., 1999). A simple restriction digestion methodology to distinguish between the BRO-1 and BRO-2  $\beta$ -lactamase types has been published (du Plessis, 2001). Of clinical relevance, isolates harboring the BRO-1 encoding gene tend to have higher transcription rates (and hence higher MICs) than BRO-2 harboring isolates (McGregor et al., 1998; Kadry et al., 2003). However, both BRO-1 and BRO-2 enzymes are inactivated by  $\beta$ -lactamase inhibitors (Francioli, 1991; Brook and Van de Heyning, 1994; Oguri et al., 2002). In any case, the choice of treatment should ideally be based upon the type of disease and condition of the patient (e.g. simple or complicated COPD; Murphy and Sethi, 2002). It is perhaps prudent for clinicians to assume that all isolates of *M. catarrhalis* are resistant to penicillin, ampicillin, amoxycillin, piperacillin and cephalothin (Livermore, 1995; Doern et al., 1996; Kadry et al., 2003). Also important for clinicians to consider is the fact that the genes for BRO-type enzymes may be easily transferred by conjugation between different species (Kamme et al., 1984; Wallace et al., 1989), a phenomenon

that may be important in the treatment of mixed infections and may occasionally result in treatment failure (Stefani et al., 1991; Hol et al., 1994; Patel et al., 1995). Interestingly, there is evidence suggests that  $\beta$ -lactamase-producing *Moraxella catarrhalis* may prevent the emergence of penicillin-resistant *Streptococcus pneumoniae* in children with recurrent acute otitis media (Joki-Erkkila et al., 2002).

*Moraxella catarrhalis* isolates may also show some inherent resistance to trimethoprim, with a recent study of 76 clinical isolates from Saudi Arabia indicating that 14.5% of isolates were resistant to this antibiotic (Kadry et al., 2003). Further, a recent survey showed that approximately 10.5% of *M. catarrhalis* isolates in Latin America (Johnson et al., 2003) and all isolates from an Indian study (Tabassum and Ahmed, 2002) were highly resistant to combination therapy using trimethoprim and sulfamethoxazole. In general, however, *M. catarrhalis* isolates are consistently susceptible to most non- $\beta$ -lactamase antibiotics including chloramphenicol, erythromycin, ciprofloxacin and gentamicin (Hoban et al., 2001; Bell et al., 2002; Hoban and Felmingham, 2002). Also, fluoroquinolones remain very active against *M. catarrhalis* (Deshpande and Jones, 2000; Esposito et al., 2000). New antibiotics for use against lower respiratory pathogens (including *M. catarrhalis*) are under constant development and testing (Boswell et al., 2002; Jones, 2002).

## Immune Responses

The immune response to bacterial pathogens is a highly complex process involving both nonspecific and specific mechanisms.

### The Nonspecific Immune Response

Nonspecific mechanisms include the presence of mucus and surfactant at pulmonary surfaces, mucociliary action, alveolar macrophage activity, and complement-mediated lysis (Toews et al., 1990). Onofrio et al. (1981) published data looking at the removal of several bacterial species from the lungs of mice, and in contrast to non-typeable *H. influenzae* and *Streptococcus* spp., found that *M. catarrhalis* was cleared relatively slowly from the lungs with a correspondingly significant increase (400X) in the numbers of granulocytes. Further, the release of neutrophil defensins (peptides exhibiting broad-spectrum antimicrobial activity released by neutrophils as part of the inflammatory process) has been shown to actually stimulate adherence of *M. catarrhalis* (conceivably involving the phase variable expression of bacterial adherence genes;

Gorter et al., 2000). Finally, *M. catarrhalis* has recently been reported to induce mast cell activation and nuclear factor kappa B-dependent cytokine (IL-6 and MCP-1) synthesis by direct contact with the mast cells (with neither bacterial lipopolysaccharide or bacterial supernatants inducing cytokine secretion; Krishnaswamy et al., 2003). The role of complement and complement resistance in *M. catarrhalis* mediated infections is discussed later.

### The Specific Immune Response

Several studies have demonstrated the presence of bactericidal antibodies in *M. catarrhalis* associated infections, including: AOM (Leinonen et al., 1981; Faden et al., 1992b; Faden et al., 1994b; Mathers et al., 1999), lower respiratory tract infections (Chapman et al., 1985; Black and Wilson, 1988; Christensen et al., 1996), pneumonia (Gabre-Selasie, 1998) and exacerbations of COPD (Bakri et al., 2002). Using whole-cell *M. catarrhalis* bacteria as coating antigen and an ELISA assay format, Goldblatt et al. (1990) showed that in the majority of cases, normal human sera tested positive for the binding of IgG<sub>1</sub>, IgG<sub>2</sub> and IgG<sub>3</sub> subclasses but not IgG<sub>4</sub>. Further, the IgG<sub>3</sub> subclass of antibody may be particularly important in *M. catarrhalis* infection (Rahman et al., 1997) as IgG<sub>3</sub>-specific antibodies are low or undetectable in children under 4 years of age (Goldblatt et al., 1990b; Chen et al., 1999a). These findings may explain the differences in colonization rates observed between children and adults (Arkwright, 1907; Faden et al., 1994a). However, studies have indicated that host Gm allotype (i.e. genetic markers present on IgG molecules) may influence the IgG subclass composition of specific antibody responses to *M. catarrhalis* outer membrane proteins (Carson et al., 1994), possibly via a complex interaction between B-cells, T-cells, HLA type, and Gm-linked V region antibody expression (Goldblatt et al., 1994). If the IgG<sub>3</sub> subclass is particularly important, and allotype variation results in differences in IgG<sub>3</sub> expression in certain individuals, then this could explain why some children suffer from chronic *M. catarrhalis* infections (e.g., chronic otitis media).

At mucosal surfaces, IgA antibody responses predominate, and specific IgA has been detected in middle ear effusions of children with otitis media (Karjalainen et al., 1991; Faden et al., 1992b; Takada et al., 1998) as well as in the saliva of healthy adult volunteers (Meier et al., 2003). Stutzmann Meier et al. (2003) indicated that *M. catarrhalis* colonization in early infancy was associated with a consistent IgA immune response directed against the outer membrane proteins. However, the development of (mucosal) IgA

antibody may not necessarily correspond to the development of (systemic) IgG antibody (Stenfors and Raisanen, 1993; Samukawa et al., 2000a). Of note, Mandrell (1992) and Troncoso et al. (2004) identified cross-reactive antigens between *M. catarrhalis*, *N. meningitidis* and *N. lactamica* and showed the presence of cross-reactive antibodies in both healthy individuals and patients convalescing from meningococcal meningitis.

### Vaccines

Currently, the efforts of many researchers in the *M. catarrhalis* field is geared towards the identification and development of specific vaccine candidates (reviewed in McMichael [2000a], McMichael [2000b] and McMichael and Green [2003]) with particular emphasis being placed on 1) the lipooligosaccharide/lipopolysaccharide (LOS/LPS) and 2) various highly expressed outer membrane proteins (OMPs; Murphy, 1989a).

Several studies have indicated that an antibody response to the LPS of *M. catarrhalis* is generated and that this response is not serotype specific but directed against common epitopes present on the LPS (Fomsgaard et al., 1991; Rahman et al., 1995; Oishi et al., 1996; Jiao et al., 2002). However, confusingly, some evidence suggests that the antibody response (in rabbits) is indeed serotype specific (Rahman and Holme, 1996). This LPS antibody response appears to be particularly directed against the oligosaccharide region of the LPS (Edebrink et al., 1994; Edebrink et al., 1995; Edebrink et al., 1996). The inoculation of nonviable *M. catarrhalis* into the middle ear bullae of guinea pigs also induces middle ear inflammation and mucoperiosteal histopathology, a type of inflammatory picture most probably mediated by bacterial outer membrane components (including the LOS; Sato, 1997). On the basis of this data, research by several groups has indicated that the LPS/LOS may be a promising vaccine candidate so long as its inherent toxicity is removed and it is linked to a protein conjugate to improve immunogenicity (Tanaka et al., 1992; Gu et al., 1998; Hu et al., 2000; Jiao et al., 2002). To complicate matters, the immunospecificity of at least three serotypes of LPS, each exhibiting some form of crossreactivity (Vanechoutte et al., 1990), may depend on differences in the lengths of the immunodominant oligosaccharides (Edebrink et al., 1996). However, no relationship between serotype and severity of infection has been noted, implying that all three of these common LOS serotypes (types A, B and C) should ideally be included in a future LPS/LOS based vaccine (Fig. 5).

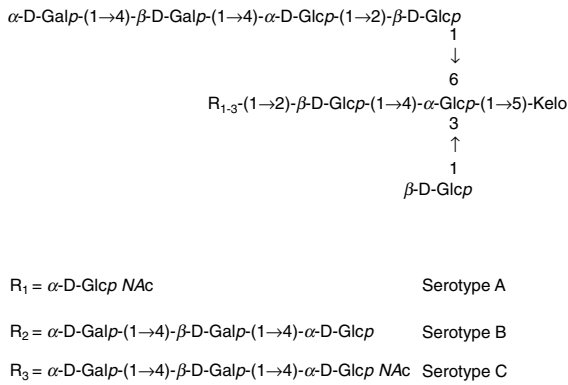


Fig. 5. Schematic structure of the lipooligosaccharide moieties from the three main serotypes (A, B and C) of *Moraxella catarrhalis*. Serotype differences are related to differences in the nature of the “R”-group. (From Verduin et al., 2002, with permission.)

Most research regarding the development of a vaccine against *M. catarrhalis* has concentrated on the identification and investigation of several major OMPs, which currently fall into roughly two distinct groups. One group includes OMPs associated with bacterial adherence, including; UspA1/2 (Helminen et al., 1994; Chen et al., 1996; Chen et al., 1999a; Aebi et al., 1997), OMP CD (Murphy et al., 1993; Yang et al., 1997; Murphy et al., 2003) and MID (Forsgren et al., 2001; Forsgren et al., 2003), while the other group includes OMPs associated with iron and fatty acid (nutrient) acquisition, LbpA, LbpB, TbpA, TbpB/B1/74-kDa protein (Bonnah et al., 1995; Bonnah et al., 1998b; Campagnari et al., 1996; Mathers et al., 1997; Bonnah and Schryvers, 1998a; Chen et al., 1999b), CopB (Helminen et al., 1993; Aebi et al., 1996; Sethi et al., 1997) and OMP E (Bhushan et al., 1994; Murphy et al., 2000).

The UspA proteins (UspA1 and UspA2) are closely related proteins that share some conserved, semiconserved, and repeat sequence domains (Aebi et al., 1997; Cope et al., 1999; Hays, 2003). Both proteins share a conserved epitope that binds the monoclonal antibody 17C7 (mab17C7), which could be useful in the progress towards a *M. catarrhalis* vaccine (Meier et al., 2002). Moreover, both proteins appear to elicit (protective) antibodies in humans (Chen et al., 1999a; Samukawa et al., 2000a; Samukawa et al., 2000b; Stutzmann Meier et al., 2003), mice (Helminen et al., 1994; McMichael et al., 1998), and guinea pigs (Chen et al., 1996). OMP CD is a protein shown to specifically bind human middle ear and nasal mucins but not salivary mucin (Reddy et al., 1997), and exhibits a high degree of sequence conservation (Yang et al., 1997;

Murphy et al., 1999). The protein has also been shown to be a target for both the mucosal and systemic immune response (Murphy et al., 2003). However, the degree of sequence conservation in OMP CD could indicate that this protein does not actually come under immunoselective pressure in vivo and may therefore not actually be a good vaccine candidate in humans. A recent study has also indicated that a 200-kDa IgD binding protein (MID) is also an adhesin (Forsgren et al., 2003), which is able to induce human B-lymphocyte (but not T-lymphocyte) activation (Gjorloff Wingren et al., 2002). This protein also appears to be conserved and is apparently present in most *M. catarrhalis* isolates (Mollenkvist et al., 2003). However, the usefulness of this MID protein as a future vaccine candidate has yet to be determined.

Studies have shown that *M. catarrhalis* expresses two lactoferrin receptors (LbpA and LbpB; Bonnah et al., 1999) and two transferrin receptors (TbpA and TbpB/OMP B1; Myers et al., 1998). Yu et al. (1999) showed that of these four iron acquisition proteins, only the LbpB and TbpB proteins show strong reactivity with acute- and convalescent-phase sera from patients with *M. catarrhalis*-associated pulmonary infections. Moreover, the antigenic heterogeneity in reactivity to the TbpB and LbpB proteins isolated from different *M. catarrhalis* strains appeared to be considerable. CopB (also known as OMP B2) is an iron-repressible 81-kDa outer membrane protein, which is largely conserved between *M. catarrhalis* isolates with discrete regions of moderate heterogeneity (Sethi et al., 1997). A copB binding monoclonal antibody (Mab10F3) was shown to enhance the pulmonary clearance of *M. catarrhalis* in a mouse model and was able to bind to 70% of *M. catarrhalis* isolates tested (Helminen et al., 1993; Aebi et al., 1998a). Moreover, significant rises in anti-copB antibody titers were observed in patients with *M. catarrhalis* infections (Helminen et al., 1995; Mathers et al., 1999; Meier et al., 2003). OMP E exhibits weak homology to the fatty acid transporter FadL of *E. coli* (Bhushan et al., 1994). The 47-kDa protein appears to be highly conserved among *M. catarrhalis* isolates (Murphy et al., 2001), and a majority of patients with chronic bronchitis were shown to have IgA antibodies to OMP E in their sputum (though surprisingly none of ten adults with *M. catarrhalis* lower respiratory tract infections demonstrated a clear-cut rise in OMP E antibody titer; Bhushan et al., 1997). Further, two *M. catarrhalis* gene knockout mutants lacking OMP E were shown to be more readily killed by normal human serum compared to the isogenic parent strains (Murphy et al., 2000). The possible role of these proteins as potential vaccine candidates is also under investigation.

Note that several groups have independently worked with the TbpB protein, such that two other names exist for TbpB, namely "B1" (Campagnari et al., 1996) and "74K protein" (Chen et al., 1999b).

### Physiology

**LIPOLYSACCHARIDE AND LIPOOLIGOSACCHARIDE** The lipopolysaccharide (LPS) and lipooligosaccharide (LOS) bacterial surface components are major virulence factors of Gram-negative bacteria, and in *M. catarrhalis* the LOS has been shown to be an important cell wall structural component (Luke et al., 2003). Biochemically, the LOS of *M. catarrhalis* isolates appears to contain no O-chain polysaccharide (Campagnari et al., 1990; Holme et al., 1999) and is of the "semi-rough" type (Fomsgaard et al., 1991). Vaneechoutte et al. (1990) were able to distinguish three different LOS types, namely types A (60%), B (30%) and C (5%). These three different types corresponded to different LOS chemical structures, all containing a common polysaccharide core but showing differences in terminal sugar groups in one of the LOS branches (Edebrink et al., 1996; Holme et al., 1999). Specifically, type B oligosaccharides contain a 4-substituted  $\alpha$ -D-glucopyranosyl residue ( $\alpha$ -D-Galp) instead of 2-acetamido-2-deoxyglucopyranosyl residues ( $\alpha$ -D-GlcpNAc). Significant levels of variation have also been reported in the lipid A region of the LPS (Kelly et al., 1996). Interestingly, the nonenteric, Gram-negative bacteria *M. catarrhalis*, *H. ducreyi*, *H. influenzae*, *N. gonorrhoeae* and *N. meningitidis* possess LOS with similar structures (Campagnari et al., 1990) and in particular a terminal trisaccharide ( $\alpha$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 2)), which is also found as the terminal part of glycolipids on the surface of human epithelial cells of mucous membranes (Holme et al., 1999). Moreover, the  $\alpha$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-Galp component of this trisaccharide has already been implicated in bacterial pathogenesis (Virji et al., 1990).

**PEPTIDOGLYCAN AND CAPSULE** The peptidoglycan layer of *M. catarrhalis* was first described by Hellio et al. (1988). This layer forms part of the *M. catarrhalis* cell envelope, and as yet very little is known regarding the role of this peptidoglycan in the immune response to *M. catarrhalis*. Keller et al. (1992) showed that *M. catarrhalis* peptidoglycan was similarly active to the peptidoglycans of *Staphylococcus aureus* and *Bacillus subtilis* in triggering the secretion of tumor necrosis factor and the tumoricidal activity of macrophages but noted that *M. catarrhalis* pep-

tidoglycan was particularly potent in its ability to induce the generation of nitrite in macrophages.

Capsules are composed of a layer of polysaccharide surrounding the bacterial cell, which are known to be important virulence factors in both Gram-positive and Gram-negative bacteria. The presence of a capsule in *M. catarrhalis* has previously been suggested (Ahmed et al., 1991), though no capsule is observed when colonies of *M. catarrhalis* grown on agar plates are examined.

**ATTACHMENT PILI AND FIMBRIAE** Pili and fimbriae are used by bacteria as attachment mechanisms, allowing them to adhere to mucosal epithelial cells. Confusingly, evidence suggests the presence of both type 4 (MePhe) and a non-type 4 class of pili (Marrs and Weir, 1990), as well as evidence for a lack of pili in *M. catarrhalis* (Ahmed et al., 1991; Rikitomi et al., 1991). These results may indicate either 1) that some strains possess pili whereas others do not, or 2) that some form of phase available expression system of pili genes occurs (as observed with *M. bovis*). Both fimbriated and nonfimbriated isolates of *M. catarrhalis* have been described, and a role for fimbriae in cellular adherence has been supported by a reduction in adherence after various treatments that denatured the fimbriae, or after binding of antifimbrial antibodies (Rikitomi et al., 1991). In a later study, increased binding to lower airway bronchial epithelium cells was observed for fimbriated strains as opposed to a nonfimbriated strain (Rikitomi et al., 1989). Fimbrial adhesion is apparently mediated via ganglioside M2 sequences in the host cell receptor (Ahmed et al., 1996).

**OUTER MEMBRANE PROTEINS** Initial studies into the outer membrane proteins of *M. catarrhalis* has revealed the presence of eight major proteins (OMPs A to H) varying in size from 21 kDa to 98 kDa, all exhibiting a relatively high degree of interstrain similarity (Bartos and Murphy, 1988; Murphy and Loeb, 1989b; Murphy, 1990). This interstrain similarity means that serological OMP profiling probably has limited use in distinguishing between individual *M. catarrhalis* isolates, though it does suggest that these proteins may be suitable as vaccine candidates. The exact function of the majority of these OMPs is as yet unclear.

OMP B2 or copB is an 81-kDa OMP that is largely conserved between *M. catarrhalis* isolates and displays discrete regions of moderate heterogeneity (Sethi et al., 1997). The expression of copB increases under iron-limiting conditions (Aebi et al., 1996), and in vitro, the protein has been shown to have the ability to bind to iron-

laden lactoferrin (Bonnah et al., 1998b). CopB therefore appears to be involved in *M. catarrhalis* iron-acquisition.

Originally considered as two distinct proteins, OMPs C and D actually represent two different yet stable forms of the same protein (protein CD; Murphy et al., 1993). Moreover, though calculated to have a molecular mass of 46 kDa, the presence of a proline-rich region means that OMP CD has an apparent mass of 60 kDa upon sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). OMP CD may play a role in cell adhesion, as it has been found to specifically bind to human middle ear and nasal (but not salivary) mucins (Reddy et al., 1997). Further, studies have indicated that the gene encoding OMP CD exhibits a high degree of homology (Hsiao et al., 1995) and that the protein is an interesting vaccine candidate (Yang et al., 1997; Murphy et al., 1999).

OMP E is an apparently highly conserved 50-kDa protein (Murphy et al., 2001) that exhibits the trimeric structure of many porins (Bhushan et al., 1994). OMP E shows weak homology to the FadL fatty acid binder and transporter of *E. coli* (Bhushan et al., 1994) and may be involved in the uptake of nutrients (e.g., fatty acids).

As well as the 8 OMPs originally identified, an extra OMP was found in the *M. catarrhalis* cell wall by Klingman and Murphy (1994). Originally called "HMW-OMP" because of its higher molecular weight (greater than 250 kDa), this OMP is most likely composed of oligomers of ca. 120–140 kDa. Later studies revealed that HMW-OMP was actually encoded by two different genes that shared a 140-amino acid region (homology = 93%) and a common monoclonal antibody epitope (Aebi et al., 1997). These genes encoded two distinct oligomeric proteins named "UspA1" (ubiquitous surface protein A1) and "UspA2" (ubiquitous surface protein A2), with calculated protein masses of only 88 kDa and 66 kDa, respectively. The apparent difference between calculated and observed mass upon SDS-PAGE is due to the formation of coiled coil structures and dimer formation in the final proteins (Hoiczky et al., 2000). Physiologically, UspA1 appears to be involved in cellular adhesion and is capable of binding fibronectin (Aebi et al., 1998b; McMichael et al., 1998), while UspA2 appears to be mainly involved in resistance to complement (Verduin et al., 1994a; Verduin et al., 1994b; Aebi et al., 1998b). Both *uspA1* and *uspA2* genes possess several common amino acid repeat motifs in their open reading frames (ORFs) and possess either a homopolymeric poly-G tract (*uspA1*) or a poly-AGAT repeat sequence (*uspA2*) in their 5' ORF promoter sequences, which may be linked to phase vari-

able gene expression (Aebi et al., 1997; Lafontaine et al., 2001). The structure and function of the *M. catarrhalis* UspA1 and UspA2 proteins share some similarity with the adhesin protein (YadA) of *Yersinia enterocolitica* (Hoiczky et al., 2000).

Iron acquisition is an important factor in bacterial survival, and *M. catarrhalis* produces both lactoferrin (LbpA and LbpB) and transferrin (TbpA and TbpB/OMP B1) receptor proteins (Yu and Schryvers, 1993; Bonnah et al., 1999). The LbpA and LbpB proteins have been sequenced and cloned (Du et al., 1998), and isogenic mutants of these genes show a reduced binding affinity for lactoferrin (Bonnah et al., 1999). The *tbpA* gene appears to be highly conserved, which contrasts with the *tbpB* gene, which is markedly heterogeneous (Myers et al., 1998). Luke et al. (1999) showed that the TbpA protein was necessary for the acquisition of iron from transferrin, yet the TbpB protein was not essential. They suggested that the function of TbpB might be to optimize the TbpA/transferrin interaction. A detailed map of peptide-peptide interactions between human transferrin and TbpB from *Moraxella catarrhalis* was recently presented by Sims and Schryvers (2003).

Recently, a 200-kDa immunoglobulin D (IgD)-binding outer membrane protein (MID) was identified by Forsgren (Forsgren et al., 2001) and later shown to be involved in both 1) the binding of *M. catarrhalis* to type II alveolar epithelial cells, and 2) the agglutination of human erythrocytes (Forsgren et al., 2003). The MID proteins from five different *M. catarrhalis* strains exhibited 71–89% similarity (Mollenkvist et al., 2003). The protein requires a tetrameric formation for optimal IgD-binding capacity (Nordstrom et al., 2002), and as with the *M. catarrhalis* *uspA1* gene, the *mid* gene also possesses several amino acid repeat motifs and a homopolymeric poly-G tract in its promoter region (Mollenkvist et al., 2003), indicating that phase variable gene expression occurs.

### Virulence Traits

According to Smith (1995), there exists five basic requirements involved in bacterial virulence, namely the ability to 1) bind, colonize and infect mucosal surfaces, 2) enter host tissues, 3) replicate in vivo, 4) interfere and possibly inhibit host defense mechanisms, and 5) damage the host organism. Much remains to be discovered about these virulence mechanisms in *M. catarrhalis*, though the presence of phase-variable virulence genes homologous to phase variable virulence genes of *H. influenzae*, has been noted (Peak et al., 1996).

**PHASE VARIABLE GENE EXPRESSION** The presence of repeat DNA sequences in the promoter regions of virulence genes of *M. catarrhalis* has been demonstrated. These sequences could influence gene expression in a phase variable manner (i.e. where differences in promoter repeat unit number in subsequent progeny bacteria affect the level of gene expression within these progeny bacteria). Seib et al. (2002) have discovered 5'-CAAC-3' repeat unit elements in the 5'-coding start region (the methylase components) of two distinct type III restriction-modification (R-M) systems. Such systems act to limit the integration of foreign DNA into the bacterial chromosome and may have an important role in the biology of *M. catarrhalis*. In a separate study, Peak et al. (1996) also discovered and assigned a role for 5'-CAAC-3' repeat sequences with virulence factor gene phase variation in *M. catarrhalis*.

The *Moraxella catarrhalis* IgD-binding outer membrane protein MID contains a homopolymeric polyguanine (poly-G) tract in its promoter region (Forsgren et al., 2001). Moreover, all isolates expressing high levels of MID had 1, 2 or 3 triplets of Gs in their poly(G) tracts, while strains not expressing MID had 4, 7, 8 or 10 Gs in their poly-G tracts (or else point mutations that prematurely terminated the protein; Mollenkvist et al., 2003). The MID protein is an adhesin allowing *M. catarrhalis* to bind to host epithelia and interestingly the UspA1 protein (another adhesin) also contains a poly-G tract (in this case in the promoter region). Though the mechanism of action may be different, phase variable expression of both of these poly(G)-tract-containing genes probably occurs. In contrast to the UspA1 protein, the similar *uspA2* gene has been shown to contain variable numbers of AGAT-repeat unit sequences in its promoter region (Aebi et al., 1997; Hays et al., 2003a), sequences that again could affect the level of gene expression for this protein, a protein implicated in complement resistance.

**BACTERIAL ADHERENCE** Bacterial adherence is a virulence factor that allows the organism to remain in situ on epithelial cells despite the action of nonspecific host defense mechanisms (e.g. the action of cilia, coughing, etc.).

However, Kyd et al. (1998) demonstrated that a nonaggregating (reduced expression of outer membrane proteins) variant of *M. catarrhalis* was less rapidly cleared in a mouse model compared to the aggregating parental strain.

Ahmed et al. (2002) showed that *M. catarrhalis* can bind to asialo-GM1 (Gg4Cer) and asialo-GM2 (Gg3Cer) gangliosides, which both contain a common GalNAc $\beta$ 1 $\rightarrow$ 4Gal $\beta$ 1 sequence. This sequence has been shown to act as a receptor attachment site for many respiratory bacteria.

Several OMPs of *M. catarrhalis* have been implicated in facilitating bacterial adherence to host epithelia, including UspA1 (Aebi et al., 1998b) and hemagglutinin (Hag) proteins (Holm et al., 2003). UspA1 (>250 kDa) is a lollipop shaped protein present on the bacterial surface, which interacts with fibronectin (McMichael et al., 1998), and binds to carcinoembryonic antigen-related cell adhesion molecules (receptors for several *Neisseria* and *Haemophilus* spp.) on the surface of human respiratory epithelial cells (Hill and Virji, 2003). The Hag protein is also a high molecular weight protein (200 kDa), which has some similarity to the IgD binding protein (MID; Fitzgerald et al., 1997) of *M. catarrhalis*, and has recently been shown to be an adhesin for cell lines derived from human lung and middle ear tissues (Holm et al., 2003). The Hag protein also has the potential to interact with both the UspA1 and UspA2 proteins as well as with host defense mechanisms (Pearson et al., 2002). Perhaps the most widely studied adhesion interaction has been that between OMP CD and host epithelia. Reddy et al. (1997) indicated that OMP CD was capable of interacting with the middle ear mucin glycoprotein (but not asialomucin), and Bernstein and Reddy (2000) indicated that the OMP CD-mucin interaction is dependent on the site where the mucin was produced, binding to mucin from the nasopharynx and middle ear but not to mucin from the tracheobronchus or saliva. More recently, Forsgren et al. (2003) demonstrated that an IgD binding protein (MID) present on the surface of *M. catarrhalis* was capable of binding to type II alveolar epithelial cells, and that mutants deficient in MID had a 50% lower adhesive capacity, even in the presence of detectable UspA1 expression. Timpe et al. (2003) recently screened for novel adhesins in *M. catarrhalis* and discovered a new potential adhesin protein (named "*M. catarrhalis* adherence protein" or "McaP"), which exhibited substantial similarity to the GDSL family of lipolytic enzymes. Finally, host factors have been shown to play a role in *M. catarrhalis* adherence to respiratory cells. In particular, attachment is increased when cells have been exposed to cigarette smoke (Kurtti et al., 1997), a response apparently not attributable to enhanced expression of host cell antigens (El Ahmer et al., 1999).

**SERUM AND COMPLEMENT RESISTANCE** The complement system is a complex series of serum proteins, which are part of the host defense mechanism against microbial pathogens (Cates, 1983; Loos and Clas, 1987). The ability of Gram-negative bacteria to withstand the effects of complement is an important virulence factor (Roantree and Rantz, 1960; Brown et al., 1983), and many *M. catarrhalis* disease-causing isolates

are apparently resistant to the effects of complement in human serum (Jordan et al., 1990; Hol et al., 1993; Hol et al., 1995). Several studies have shown the importance of complement resistance to *M. catarrhalis* pathogenicity (Brorson et al., 1976; Soto-Hernandez et al., 1989; Murphy et al., 1997), with isolates from the upper respiratory tract of children or healthy adults tending to be complement sensitive, whilst isolates from children and adults with lower respiratory tract infections tend to be complement resistant (Hol et al., 1995; Verduin et al., 2002).  $\beta$ -Lactamase production (another virulence factor) does not appear to be related to complement resistance in *M. catarrhalis* (Schmitz et al., 2002), possibly reflecting the plasmid borne nature of  $\beta$ -lactamase production or the multifactorial nature of complement resistance. Our knowledge regarding complement resistance mechanisms in *M. catarrhalis* is as yet limited. Prellner (1980) provided evidence for high level binding of C1q complement component to *M. catarrhalis*, a phenomenon that would render the organism sensitive to the antibacterial activity of complement. Research by Verduin et al. (1994a) showed that at least one resistance mechanism involves inhibition of formation of the membrane attack complex of complement and the binding of vitronectin (a natural inhibitor of complement found in serum) to the UspA2 protein present on the surface of *M. catarrhalis* (Verduin et al., 1994b; McMichael et al., 1998). An *M. catarrhalis* mutant lacking the *uspA2* gene was found to be sensitive to complement-mediated killing, while the parent isolate was resistant (Aebi et al., 1998b). Further, UspA2 protein shares similarity with the *H. ducreyi* DsrA (*ducreyi* serum resistance protein A), which has also been shown to be involved in serum resistance (Cole et al., 2002). A *copB* gene knockout *M. catarrhalis* mutant was used by Helminen et al. (1993) to show that reduced expression of the CopB (or OMP B2) iron acquisition protein decreased serum resistance and survival in vivo. Confusingly, however, Furano and Campagnari (2003) recently indicated that constitutive expression of iron-regulated proteins (via inactivation of the ferric iron uptake regulator gene *fur*) actually increases the susceptibility of *M. catarrhalis* to the bactericidal activity of normal human sera and suggested that this most probably occurs because of the upregulated expression of OMPs, increasing the presence of multiple antigenic targets for human antibody binding and hence complement activation. One further study of *M. catarrhalis* OMP expression indicated that OMP E (a putative fatty acid transporter) may be involved in serum sensitivity (Murphy et al., 2000). In 2000, Zaleski et al. (2000) implicated the *M. catarrhalis* LOS in complement resis-

tance, as inactivation of the *galE* gene, which encodes a UDP-glucose-4-epimerase (involved in the biosynthesis of LOS) resulted in enhanced serum susceptibility of the mutant. Interestingly, similarities between *M. catarrhalis* and some more thoroughly studied *Neisseria* spp. (e.g., *N. gonorrhoea* and *N. meningitidis*), with respect to OMPs and LOS, may provide further clues as to the mechanisms by which complement resistance is mediated in *M. catarrhalis*.

Finally, several studies have indicated that complement resistant and complement sensitive isolate of *M. catarrhalis* may actually represent two different subspecies (Verduin et al., 2000; Bootsma et al., 2000a; Bootsma et al., 2000b).

### Animal Models and Virulence

One of the earliest reports of an animal model for *M. catarrhalis* was published in 1989 by Doyle et al. (1989), who inoculated *M. catarrhalis* into the middle ear of chinchillas and gerbils facilitating the production of an effusion (containing no live bacteria after 24 h). Further work by Chung et al. (1994) also indicated that the chinchilla model may be useful in studying *M. catarrhalis* infection (specifically otitis media). The majority of *M. catarrhalis* animal model studies have however, focused on the mouse model of infection. Verghese et al. (1990) studied the pulmonary clearance and phagocytic cell response of *M. catarrhalis* in a murine model. In this endotracheal challenge model, a high influx of polymorphonuclear leucocytes into the lungs was observed, with the bacterium being cleared within 24–48 h (though mice with a C5 complement component deficiency showed a minor delay in clearance). Another mouse model utilized transoral inoculation of the lungs (using surgery to expose the trachea) to evaluate the bacterial-lung interaction and observe any pathological changes (Unhanand et al., 1992). Using essentially the same model, MacIver et al. (1993) showed that an enhanced clearance of bacteria from the lungs of infected mice could be obtained after immunization with *M. catarrhalis* derived outer membrane vesicles. Another mode of infection was utilized by Kyd et al. (1999), who directly inoculated killed bacteria into the Peyer's patches of mice followed by an intratracheal booster inoculation. Enhanced clearance of bacteria was observed in the lungs, which correlated to higher levels of specific IgG and IgA in both serum and bronchoalveolar lavage fluid. Hu et al. (Hu et al., 1999; Hu et al., 2000) as well as other researchers (Hou et al., 2002; Jiao et al., 2002) have had some success in developing an inhalation model and using it to assess various immunization strategies. On the whole however, there is still some debate over precisely which



is the best animal model for mimicking *M. catarrhalis*-mediated human infections (Melhus and Ryan, 2003). For example, several recent pathogenesis studies used the rat instead of the mouse as a model of infection (Jecker et al., 1999; Westman et al., 1999; Caye-Thomasen et al., 2000; Kyd and Cripps, 2000), while the development of PCR-mediated detection methodologies have focused on the chinchilla model (Bakaletz et al., 1995; Post et al., 1996; Aul et al., 1998).

## Final Comments

*Moraxella catarrhalis* is now firmly established as a pathogen in its own right, generally facilitating upper respiratory tract disease in children and lower respiratory tract disease in adults. The carriage and spread of *M. catarrhalis* isolates in daycare centers, nursery schools, and in the nosocomial setting has been established, and almost all clinical isolates are now resistant to penicillin-based antibiotics. Currently however, relatively little is known about the mechanisms facilitating virulence in this pathogen. Vaccines against the more prevalent respiratory bacterial pathogens *S. pneumoniae* and *H. influenzae* are presently in an advanced state of development, which if successful, could result in an increase in infections attributed to *M. catarrhalis* in the future (as the organism seeks to fill the empty niche left after vaccination against these two pathogens). With this in mind, further research into a vaccine against *M. catarrhalis*, as well as the virulence mechanisms attributable to this organism, are warranted.

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## *Legionella* Species and Legionnaires' Disease

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### **Introduction and History**

*Parts of this chapter were modified from the same chapter of the previous book edition, written by William Pasculle.*

Legionnaires' disease is an acute pneumonic illness caused by Gram-negative bacilli of the genus *Legionella*. Pontiac fever is a febrile, non-pneumonic, systemic illness closely associated with, if not caused by, *Legionella* spp.

Legionnaires' disease was first recognized as a distinct clinical entity when it caused an epidemic of pneumonia at an American Legion convention in Philadelphia in 1976; 221 people were affected, and 34 died (Fraser et al., 1977). Despite intensive laboratory investigation, the cause of the outbreak went undetected for many months. This mystery provoked considerable fear and widespread speculation about the cause, including claims that it was a government plot, a germ warfare experiment, and a toxin. A thorough epidemiologic investigation determined that the disease was most likely airborne, and focused primarily at one convention hotel. The inability to determine the cause of the outbreak confounded politicians and scientists who had thought that there were no new infectious diseases to be discovered. There was enough national and political concern to prompt two independent congressional investigations of the outbreak. About six months later, two investigators at the United States Centers for Disease Control and Prevention (CDC), Joseph McDade and Charles Shepard, announced that they had discovered the etiologic agent, a fastidious Gram-negative bacillus (McDade et al., 1977). Because of the historical association with the American Legion convention, this disease is now called "Legionnaires' disease," and the etiologic agents belong to the family Legionellaceae, with *L. pneumophila* being the agent responsible for the 1976 Philadelphia epidemic.

Use of an antibody test for the disease showed that several prior unsolved outbreaks of pneumonia had been Legionnaires' disease, including epidemics investigated in the 1950s (Osterholm et al., 1983; Stout and Yu, 1997; Broome and

Fraser, 1979; Winn, 1988; Breiman and Butler, 1998). An unsolved epidemic of a nonpneumonic febrile illness was also found to be due to exposure to *Legionella* bacteria; this illness was termed "Pontiac fever," after the city where this had occurred (Glick et al., 1978; Kaufmann et al., 1981). In addition, saved culture isolates from the 1940s through the 1960s were found to be *Legionella* bacteria although at the time they had been thought to be rickettsial agents (Tatlock, 1944; 1947; 1982; Bozeman et al., 1968; Hébert et al., 1980a). Thus both the organism and the disease had previously been studied decades before but had been forgotten. Ever since the 1976 outbreak in Philadelphia, ([http://www.q-net.net.au/~legion/Legionnaire's\\_Disease\\_CONTENTS.htm](http://www.q-net.net.au/~legion/Legionnaire's_Disease_CONTENTS.htm) {Legionnaires' disease}) has taken on an undeserved reputation of a highly fatal and common disease.

### **Clinical and Public Health Aspects of Legionnaires' Disease**

#### **Clinical Presentation**

Legionnaires' disease presents clinically as pneumonia, with features indistinguishable from other common forms of bacterial pneumonia, such as pneumococcal pneumonia (Granados et al., 1989; Roig et al., 1991; Sopena et al., 1998; Tan et al., 2000; Edelstein, 1993a; Edelstein and Meyer, 1995b). There is some suggestion that a combination of factors such as diarrhea, hyponatremia, and increased serum creatine kinase is more consistent with Legionnaires' disease than other pneumonic diseases, but no study has shown this unequivocally. Thus the clinical presentation generally consists of fever, fatigue, often headache or muscle aches and cough. Chest pain, diarrhea, confusion, shaking chills, and shortness of breath also may be seen. The chest roentgenogram usually demonstrates alveolar filling, focal infiltrates, and lung consolidation with or without pleural effusions.

Extrapulmonary infection occurs rarely, either as disseminated infection in patients with pneu-

monia or very rarely as isolated primary infection (Edelstein, 1993a; Stout and Yu, 1997). Isolated disease of prosthetic heart valves, respiratory sinuses, and open wounds have all been reported. Pleural empyema, myocarditis, meningitis, encephalitis, pericarditis, vascular shunt infections, peritonitis and colitis have all been documented to very rarely occur during the course of pneumonia (Edelstein and Meyer, 1995b).

### Treatment

Legionnaires' disease is treated with macrolide, tetracycline, or fluoroquinolone antimicrobial agents (Edelstein, 1995a; 1998). All of these agents concentrate within macrophage lysosomes. The drugs of choice to treat mild disease in community-acquired pneumonia include erythromycin, doxycycline, azithromycin and levofloxacin. For severely ill patients, or immunocompromised ones, either azithromycin or levofloxacin are the drugs of choice (Edelstein, 1995a; 1998). Antimicrobial agents that are ineffective include all  $\beta$ -lactam agents and penems, aminoglycosides, glycopeptides and chloramphenicol. The response to treatment depends on the patient's age, underlying diseases, degree of pulmonary involvement, the timing of treatment in relation to disease onset, and severity of disease. Untreated disease is fatal in 5–80% of patients, depending on the above factors; previously healthy people with minimal disease have the best outcome, and otherwise ill or immunocompromised patients with extensive pneumonia the worst outcome. Prompt specific therapy reduces the fatality rate by two- to sixfold. The duration of therapy, depending on the agent used and the presence of immunosuppression, ranges from 3–21 days; patients with endocarditis or cavitating pneumonia may require longer courses of therapy.

### Epidemiology

Legionnaires' disease causes both sporadic and epidemic pneumonia worldwide. In the United States, the sporadic form of the disease causes around 10,000 cases of pneumonia per year in adults requiring hospitalization for pneumonia, and perhaps the same number of cases of pneumonia in adults not requiring hospitalization for pneumonia (Marston et al., 1997; Breiman and Butler, 1998). Overall this represents approximately 1–4% of all adult pneumonias in the United States. However in its epidemic form, the disease can be responsible for a large number of cases over a short time period, for example 226 people developed Legionnaires' disease, 18 of whom died, over five days during an epidemic

that occurred at a (<http://www.eurosurv.org/1999/990401.html>{flower show}) in the Netherlands. Such epidemics are fortunately rare, but they do remind us that disease prevention through the use of proper engineering and construction controls and of rapid disease recognition through laboratory testing is important, and that knowledge of the important factors responsible for disease outbreaks is lacking.

Major risk factors for community-acquired Legionnaires' disease include administration of glucocorticosteroid medications and other forms of immunosuppression, cigarette smoking, chronic severe renal failure, age greater than 50 years, AIDS, hematologic malignancies, lung cancer and male gender (Marston et al., 1994). Alcohol abuse may or may not be a significant risk factor (Broome and Fraser, 1979; Marston et al., 1994). The same risk factors seem to apply for nosocomial acquisition, as well as exposure to a known source of the organism (Haley et al., 1979; Shands et al., 1985; Carratala et al., 1994; Joseph et al., 1994). Surgery, or more probably general anesthesia, also has been a risk factor in some nosocomial epidemics of Legionnaires' disease (Serota et al., 1981; Korvick and Yu, 1987).

The incubation period of the disease is about 2–10 days (Breiman and Butler, 1998). The disease is spread primarily via aerosols of contaminated water, although microaspiration also may be an important mode of spread in nosocomial disease (Breiman and Butler, 1998; Blatt et al., 1993; Venezia et al., 1994). The sources of *Legionella*-containing aerosols are usually water-cooled heat rejection equipment such as air-conditioning cooling towers, whirlpool spas, sink taps and shower heads (Brundrett, 1992). Virtually any device that can create an aerosol of warm water can be a disease source, including such equipment as ice machines, vegetable misters, and compressed oxygen humidifiers. The bacterium multiplies best in water at temperatures between about 25–42°, and so to contain high concentrations of the bacterium, the contaminated water must be heated either naturally or by mechanical equipment. A major source of such warm water is a water heater, which often contains several different thermoplanes that do not mix well, leaving pockets of relatively cool water.

Strain typing is often crucial when investigating epidemics of Legionnaires' disease (Mahoney et al., 1992; Mamolen et al., 1993; Pruckler et al., 1995b; Whitney et al., 1997; Brown et al., 1999; Fiore et al., 1998; Joseph et al., 1996; Hunt et al., 1991). These results most often are used to determine the environmental source of an outbreak, as multiple same-species and serogroup culture-positive environmental sites are usually found during outbreak investi-

gations. Generally only one of these sites is linked to the outbreak. A large variety of typing methods can be used in this circumstance. The first and most widely used one was subtyping of *L. pneumophila* serogroup 1 using a monoclonal antibody typing panel (Joly et al., 1986b). Unfortunately some of the hybridomas for making the antibodies are no longer available, so the typing scheme cannot be used as initially proposed. However, enough of the reagents are still available and are used commonly in outbreak investigations by national public health laboratories. Monoclonal typing schemes for other *L. pneumophila* serogroups have been developed, and newer antibody panels have been made to replace the original *L. pneumophila* serogroup 1 panel (Helbig et al., 1994; Lück et al., 1991; 1992; 1995a). Plasmid typing was never very useful for strain subtyping, as many epidemic strains lacked plasmids; even when plasmids were present, the method was slow and technically demanding (Edelstein et al., 1986). However, for plasmid-containing strains there is good correlation with monoclonal typing results. Analysis of chromosomal DNA polymorphism using frequently cutting restriction endonucleases also was found to be useful, but technically demanding and sometimes difficult to interpret. Pulse-field gel electrophoresis (PFGE) of chromosomal DNA cut with infrequent cutters supplanted the earlier molecular typing methods, and has been found to generally be reliable (De and Harrison, 1999; Riffard et al., 1998b; Gautom, 1997; Lück et al., 1995b). Other molecular methods that have been used for this purpose include ribotyping, repetitive element polymerase chain reaction (PCR), and infrequent-restriction site PCR (Fry and Harrison, 1998; Stout et al., 1988; Saunders et al., 1990; Tram et al., 1990; Van Belkum et al., 1993; Pruckler et al., 1995b; Grattard et al., 1996; Van Belkum et al., 1996; Bansal and McDonell, 1997; Miyamoto et al., 1997; Riffard et al., 1998b; De and Harrison, 1999). The two most promising methods appear to be random arbitrarily primed PCR and amplified fragment length polymorphism (AFLP). The AFLP method appears to be the most reproducible, and with proper standardization, this method might be used as a standard typing scheme (Jonas et al., 2000).

### General Description of the Genus

The Legionellaceae are mesophilic, assaccharolytic, obligately aerobic Gram-negative bacilli. Amino acids rather than carbohydrates are used as an energy source. *Legionella pneumophila*, *L. micdadei*, and probably many other, if not all, *Legionella* spp. are facultative parasites of many eukaryotic cells, including some free-living

amoebae. The bacterial length is highly variable, depending upon growth conditions, stage of growth, and whether the bacterium is grown in eukaryotic cells or extracellular environments. When grown on solid media the bacterium is usually a rod 5–40 µm in length, with a cell width of 0.3–0.9 µm; bacteria incubated on plates for many days may become shorter and motile. When grown to early log phase in broth, the bacterium is usually 5–10 µm in length and single-celled, but some may be filamentous. When grown to late stationary phase ( $OD_{600} > 1.8$ ) in broth many of the bacteria become coccobacillary and highly motile, whereas some forms are filamentous (Pine et al., 1979; Byrne and Swanson, 1998). Bacteria grown in amoebae, macrophages, or macrophage-like cell lines are short coccobacilli. All are weakly catalase-positive. The oxidase reaction is usually weak and may be negative. They are nonsporeforming and unencapsulated. All but a few species are motile by means of one to three polar or lateral flagellae, although motility may be lost. L-Cysteine is required for growth, and iron is required for initial isolation from the environment or clinical specimens; iron-free cultivation of multiple-passage isolates has been reported. The pH and temperature optima for in vitro growth are 6.8–7.0 and 25–42°C, respectively, with optimal growth occurring in vitro between 35 and 37°C. The growth optimum in nature is unknown, but may have a very wide range (10°–45°C). Branched chain fatty acids are the predominant cellular fatty acids. Cellular ubiquinones contain 9–14 isoprene units.

The G+C content of the *Legionella* genus is 38–52 mol%. The size of the *L. pneumophila* genome is approximately 3.9 Mb (Bender et al., 1990). Methods are available for constructing a genetic map of the *Legionella* chromosome (Mintz and Zou, 1992a), and current efforts are in determining [\[genome3.cpmc.columbia.edu/~legion/index\]](http://genome3.cpmc.columbia.edu/~legion/index) the entire sequence of the *L. pneumophila* strain Philadelphia-1 genome. Among the three means of bacterial genetic exchange, conjugation and transformation are known to function within the *Legionella* genus (Chen et al., 1984; Dreyfus and Iglewski, 1985; Segal et al., 1998b; Stone and Abu Kwaik, 1999a; Vogel et al., 1998). Thus far, there have been no reports of transduction or bacteriophage in the legionellae. Factors known to influence DNA exchange between legionellae are restriction endonucleases (e.g., *LpnI*), Dam-methylation, and RecA (Chen et al., 1986; Hamablet et al., 1989; Lema and Brown, 1996; Zhao and Dreyfus, 1990). Plasmids have been detected in some strains of *Legionella*, including representatives of *L. pneumophila* and at least five other species



(Aye et al., 1981; Brown et al., 1982; Castellani Pastoris et al., 1987; Johnson and Schalla, 1982; Lopez de Felipe and Martinez-Suarez, 1991; Maher et al., 1983; Mansfield et al., 1997; Melado et al., 1986; Mikesell et al., 1981; Mintz et al., 1992c; Nolte et al., 1984; Tompkins et al., 1987). The *Legionella* plasmids occur in clinical and environmental isolates, encompass multiple incompatible groups, and range in size from 21–95 MDa. Some *L. pneumophila* plasmids are either self-transmissible or mobilizable upon bacterial conjugation (Lopez de Felipe, 1993; Mintz et al., 1992c; Tully, 1991). Although a 36-MDa *L. pneumophila* plasmid confers resistance to ultraviolet light, the majority of *Legionella* extrachromosomal elements have not been directly linked to any particular function, including aspects of intracellular infection (Mintz et al., 1992c; Tully, 1991).

### Taxonomy

The genus *Legionella* was initially defined by Brenner and colleagues who examined several isolates of the newly discovered Legionnaires' disease bacillus and concluded that they represented a new family, genus and species (Brenner et al., 1979). Since then, the genus has been redefined twice to take into account the ever-increasing number of species and subspecies. Oligonucleotide cataloging of *Legionella* 16S rRNA has demonstrated that the legionellae are not closely related to other groups of organisms, but are closely related to each other at the ribosomal level (Ludwig and Stackebrandt, 1983; Fry et al., 1991). They are classified within the  $\gamma$ -subdivision of the Proteobacteria, within the “[Legionellaceae group]” (Ludwig and Stackebrandt, 1983).

The *Coxiella* group is included in the Legionellaceae group, and includes *Coxiella* and *Rickettsiella*. The main member of the Legionellaceae group is the Legionellaceae family. In addition to *Legionella*, the Legionellaceae family contains the *Amoeba proteus* symbiotic bacterium, *Fluoribacter* (see below), *Sarcobium*, *Tatlockia*, and a large number of unclassified bacteria that are amoebal pathogens.

Forty-two *Legionella* species have been validly published (Table 1). In addition, more than {20 novel species} have been given names, and at least 10 more without names are not yet validly published (Hookey et al., 1996; Ratcliff et al., 1997; 1998; LoPresti et al., 1998b; Riffard et al., 1998a; Benson et al., 1989; 1990; 1991; 1996a; 1996b; Dennis et al., 1993; Thacker et al., 1988a; 1989; 1991; 1992; Verma et al., 1992; Wilkinson et al., 1987b; 1988; Brenner, 1986; 1987; Bercovier et al., 1986; Brenner et al., 1979;

1980; 1985; Gorman et al., 1985; Campbell et al., 1984; Orrison et al., 1983; Cherry et al., 1982; Edelstein et al., 1982b; McKinney et al., 1981; Hébert et al., 1980a; 1980b; Morris et al., 1980). Twenty *Legionella* spp. have been reported to cause human pneumonia, with the remainder having been isolated only from water sources (Tables 1 and 2). All of the *Legionella* species that have been isolated from clinical sources also have been isolated from the environment, with the exceptions of *L. hackeliae* and *L. tucsonensis*.

Several *Legionella* spp. are apparent endosymbionts of amoebae. So far most of these have not been cultured outside of their protozoal host, making their characterization difficult. These include *Legionella lytica* (also called “*L. lyticum*”), previously known as *Sarcobium lyticum*, and a number of unnamed species known generically as “LLAP” (*Legionella*-like amoebal pathogen; Hookey et al., 1996; Adeleke et al., 1996). A recent taxonomic study indicated that there are at least three distinct *Legionella* spp. among the LLAPs (Birtles et al., 1996). Unpublished work suggests {species names} for such organisms.

### Alternative Genus Names

A small number of investigators have proposed alternative genera for some of the *Legionella* species. Garrity and Brown suggested that *L. micdadei* be renamed *Tatlockia micdadei*, and that *L. bozemanii* be renamed *Fluoribacter bozemanii* (Garrity et al., 1980; 1982; Brown et al., 1981). In addition, they suggested that other blue-white autofluorescent *Legionella* species be placed into the *Fluoribacter* genus, which at the time included *L. dumoffii* (*F. dumoffii*), and *L. gormanii* (*F. gormanii*). These suggestions were based on the less than 25% DNA homology (determined by DNA-DNA hybridization) between these different *Legionella* species and *L. pneumophila*. In addition, the same group suggested that some members of *L. pneumophila* serogroup 5 be placed into a new, unnamed species, based on low homology to other members of serogroup 5 (Garrity et al., 1982).

Using multilocus enzyme electrophoresis, Selander and colleagues found multiple distinct clones within a large collection of *L. pneumophila* isolates; two of these were distant enough from the others to warrant possible classification as novel *Legionella* species (Selander et al., 1985). Brenner and colleagues (Brenner et al., 1988) confirmed that these two clones were distinct, forming two distinct DNA hybridization groups. Although highly related (88–98%) to each other, these groups were only distantly related (40–50% under stringent conditions) to

Table 1. Original descriptions and sources of the *Legionella* spp.

<i>Legionella</i> spp.	Publication year	Source	Type strain/ATCC no.	Current no. serogroups	Reference
<i>adelaidensis</i>	1991	Water	1762-AUS-E/49625	1	Benson et al., 1991
<i>anisa</i>	1985	Water <sup>a</sup>	WA-316-C3	1	Gorman et al., 1985
<i>birminghamensis</i>	1987	Human	1407-AL-H/43702	1	Wilkinson et al., 1987
<i>bozemanii</i>	1980	Human	WIGA/33217	2	Brenner et al., 1980
<i>brunensis</i>	1988	Water	441-1/43878	1	Wilkinson et al., 1988
<i>cherrii</i>	1985	Water	ORW/35252	1	Brenner et al., 1985
<i>cincinnatiensis</i>	1988	Human	72-OH-H/43753	1	Thacker et al., 1988a
<i>dumoffii</i>	1980	Human	NY 23/33279	1	Brenner et al., 1980
<i>erythra</i>	1985	Water	SE-32A-C8	2	Brenner et al., 1985
<i>fairfieldensis</i>	1991	Water	1725-AUS-E/49588	1	Thacker et al., 1991
<i>feeleyi</i>	1984	Water <sup>a</sup>	WO-44C/35072	2	Herwaldt et al., 1984
<i>geestiana</i>	1993	Water	1308/49504	1	Dennis et al., 1993
<i>gormanii</i>	1980	Water <sup>a</sup>	LS-13/33297	1	Morris et al., 1980
<i>gratiana</i>	1989	Water	Lyon 8420412/49668	1	Bornstein et al., 1989a
<i>hackeliae</i>	1985	Human	Lansing 2/35250	2	Brenner et al., 1985
<i>israelensis</i>	1986	Water	Bercovier 4/43119	1	Bercovier et al., 1986
<i>jamestownensis</i>	1985	Water	JA-26-G1-E2/35298	1	Brenner et al., 1985
<i>jordanis</i>	1982	Water <sup>a</sup>	BL-540/33623	1	Cherry et al., 1982
<i>lansingensis</i>	1992	Human	1677-MI-H/49751	1	Thacker et al., 1992
<i>londiniensis</i>	1993	Water	1477/49505	1	Dennis et al., 1993
<i>longbeachae</i>	1981	Human	Long Beach 4/33462	2	McKinney et al., 1981
<i>"lytica"</i>	1996	Human	PCM 2298/NA	NA	Hookey et al., 1996
<i>maceachernii</i>	1985	Water <sup>a</sup>	PX-1-G2-E2/35300	1	Drozanski, 1991
<i>micdadei</i>	1980	Human	TATLOCK/33218	1	Brenner et al., 1985
<i>moravica</i>	1988	Water	316-36	1	Hébert et al., 1980b
<i>nautarum</i>	1993	Water	1224/49506	1	Wilkinson et al., 1988
<i>oakridgensis</i>	1983	Water <sup>a</sup>	OR-10/33761	1	Dennis et al., 1993
<i>parsiensis</i>	1985	Water <sup>a</sup>	PF-209C-C2/35299	1	Orrison et al., 1983
<i>pneumophila</i>	1979	Human	Philadelphia 1/33152	1	Brenner et al., 1985
<i>quateirensis</i>	1993	Water	1335/49507	16	Brenner et al., 1979
<i>quintlivanii</i>	1989	Water	1442_AUS-E/43830	1	Dennis et al., 1993
<i>rubrilucens</i>	1985	Water	WA-270A-C2/35304	2	Benson et al., 1989
<i>sainthelensi</i>	1984	Water <sup>a</sup>	Mt. St. Helens 4/35248	1	Brenner et al., 1985
<i>sainticrucis</i>	1985	Water	SC-63-C7/35301	1	Campbell et al., 1984
<i>shakespearei</i>	1992	Water	214/49655	1	Brenner et al., 1985
<i>spiritensis</i>	1985	Water	Mount St. Helens 9/35249	1	Verma et al., 1992
<i>steigerwaltii</i>	1985	Water	SC-18-C9/35302	2	Brenner et al., 1985
<i>taurinensis</i>	1999	Water	Turin 1 no. 1/700508	1	Brenner et al., 1985
<i>tucsonensis</i>	1989	Human	1087-AZ-H/49180	1	Brenner et al., 1985
<i>wadsworthii</i>	1982	Human	Wadsworth 81-716A/33877	1	LoPresti et al., 1999
<i>waltersii</i>	1996	Water	2074-AUS-E/51914	1	Thacker et al., 1989
<i>worsleiensis</i>	1993	Water	1347/49508	1	Edelstein et al., 1982a
					Benson et al., 1996b
					Dennis et al., 1993

<sup>a</sup>Isolates from humans have also been reported

*L. pneumophila*, with which most isolates share common antigens. Because these organisms can only be differentiated from each other in most cases by DNA hybridization studies rather than phenotypic testing, it was proposed that the species *L. pneumophila* be divided into three subspecies: *L. pneumophila* subsp. *pneumophila*, *L. pneumophila* subsp. *fraseri* and *L. pneumophila* subsp. *pascullei*. Neither the creation of the subspecies nor the use of genera other than *Legionella* has been widely adopted. Most authorities now accept the use of a single genus, and no subspecies within *L. pneumophila*. Some

bacterial strains deposited in culture collections, or nucleic acid sequences deposited in GenBank use these epithets.

### Culture Media

*Legionella pneumophila* grows best on rich media containing yeast extract, L-cysteine, iron and organic buffers. Buffered charcoal yeast extract medium supplemented with  $\alpha$ -ketoglutarate (BCYE $\alpha$ ) is the primary culture medium used by most clinical laboratories. The medium pH must be 6.80–6.90, and the buffer

Table 2. Reports of clinical isolation of the *Legionella* spp.

<i>Legionella</i> spp.	References	<i>Legionella</i> spp.	References
<i>anisa</i>	Bornstein et al., 1989b Fallon and Stack, 1990 Thacker et al., 1990	<i>hackeliae</i>	Wilkinson et al., 1985b
<i>birthingamensis</i>	Wilkinson et al., 1987	<i>jordanis</i>	Thacker et al., 1988b Baty et al., 1997
<i>bozemanii</i>	Parker et al., 1983 Parry et al., 1985 Fukumoto et al., 1986 Jaeger et al., 1988 Humphreys et al., 1992 Taylor and Albrecht, 1995 Harris et al., 1998 Bozeman et al., 1968 Cordes et al., 1979 Lewallen et al., 1979 Thomason et al., 1979	<i>lansingensis</i>	Thacker et al., 1992
<i>cincinnatiensis</i>	Thacker et al., 1988a Jernigan et al., 1994	<i>longbeachae</i>	Bibb et al., 1981 McKinney et al., 1981 Lam et al., 1982 Lim et al., 1989 Steele, 1989 Korman et al., 1998 Koide et al., 1999 Rowbotham, 1993 Wilkinson et al., 1985a Merrell et al., 1991 Thomas et al., 1992
<i>dumoffii</i>	Lewallen et al., 1979 Brenner et al., 1980 Molnar et al., 1984 Badham et al., 1985 Edelstein and Pryor, 1985 Joly et al., 1986a Tompkins et al., 1988 Fujita et al., 1989 Fang et al., 1990b	<i>“lytica”</i>	Aronson et al., 1981 Donegan et al., 1981 Wing et al., 1981 Wing et al., 1982 Bäck et al., 1983 Dowling et al., 1983 Griffin et al., 1983 Myerowitz et al., 1979 Fang et al., 1987 Tatlock, 1944 Tang et al., 1985
<i>feelei</i>	Thacker et al., 1985 Palutke et al., 1986 Misra et al., 1987 Schousboe et al., 1995 LoPresti et al., 1998a	<i>micdadei</i> <sup>a</sup>	LoPresti et al., 1997 McDade et al., 1977 Benson et al., 1990 Thacker et al., 1989 Edelstein et al., 1982a
<i>gormanii</i>	Griffith et al., 1988 Ephros et al., 1989 Townsend et al., 1994	<i>oakridgensis</i>	
		<i>parsiensis</i>	
		<i>pneumophila</i> <sup>a</sup>	
		<i>sainthelensi</i>	
		<i>tucsonensis</i>	
		<i>wadsworthii</i>	

<sup>a</sup>Only representative reports.

can be either 3-[N-morpholino] propane-sulfonic acid (MOPS) or N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) although MOPS is more stable for optimal growth of the organism (Edelstein and Edelstein, 1993c). The  $\alpha$ -ketoglutarate, is a growth stimulant (Pine et al., 1986), and is used in the BCYE medium of almost all clinical microbiology laboratories, but some research investigators omit it. The charcoal inactivates toxic peroxides produced during autoclaving of the yeast extract (Hoffman et al., 1983), and toxic compounds present in agar (Rogers et al., 1993). Alternatively, the yeast extract can be filter sterilized, facilitating the production of a clear solid medium made without charcoal, although such media do not support growth as well as does BCYE $\alpha$  (Edelstein et al., 1982c). The BCYE $\alpha$  medium can be made selective for *L. pneumophila* by the addition of cephamandole (4 mg/liter), polymyxin B (80,000 U/liter) and anisomycin (80 mg/liter); this medium is known as “BMPA” or “PAC” medium (Edelstein, 1981). Substituting the

antifungal agent natamycin for the very expensive anisomycin is more economical (Edelstein and Edelstein, 1996). Another potential antifungal agent is fluconazole (Lin et al., 1999). Because some *Legionella* spp. may be sensitive to  $\beta$ -lactam drugs, the substitution of vancomycin for cephamandole is used to make another selective medium, called “PAV”; the addition of glycine to this medium inhibits many environmental bacteria, and is known as “modified Wadowsky Yee” (MWY) medium (Wadowsky and Yee, 1981; Edelstein, 1982a). Although these nonselective and selective growth media have been optimized for the growth of *L. pneumophila*, it is quite possible that some other *Legionella* spp. will grow better on different medium formulations.

When cultured from spleen tissue, some *Legionella* spp., such as *L. micdadei*, may grow better in the presence in 1% albumin (Morrill et al., 1990); dilution of the spleen tissue removes the benefit of the albumin. Some research investigators also include albumin in their medium.

A variety of broth media can be made for the growth of *L. pneumophila*. The optimal growth medium is the liquid equivalent of BCYE $\alpha$  medium, made without agar; this charcoal-containing broth is termed "BCYE $\alpha$  broth". Supporting the growth of a wide range of *Legionella* spp., BCYE $\alpha$  broth is far better than other broth formulations at cultivating the bacteria in the presence of inhibitors, such as spleen tissue; it also is superior to other broths for recovery of frozen *Legionella* bacteria (P. Edelstein, unpublished observation). However, the inclusion of charcoal precludes visual and spectrophotometric estimation of bacterial growth in the broth, and the charcoal also inhibits many antimicrobial agents. For this reason, BYE $\alpha$  broth (made by filter sterilization of the yeast extract, to which the other autoclaved medium components are added) is used for a variety of reasons, including antimicrobial susceptibility testing and for estimating growth of the bacterium when the charcoal is not desired (Edelstein and Edelstein, 1989b; Edelstein et al., 1989c; Ristroph et al., 1980; Barker et al., 1986). The addition of ACES (rather than MOPS) buffer to BYE $\alpha$  broth may enhance the virulence of *L. pneumophila* (Ross et al., 2000).

### Genus and Species Identification

Identification of *Legionella* spp. to the genus level is usually quite easy, as is identification of *L. pneumophila*. This is because the combination of L-cysteine dependence, colonial morphology and growth rate is very specific for *Legionella* spp., and because a very sensitive and specific monoclonal antibody for *L. pneumophila* is available for identification. The serological method was initially used to identify isolates beyond genus level. However, it soon became apparent that many species share antigenic epitopes so that even within a species, serological identification may be misleading. Very simple to complex tests that require sophisticated analytical instruments have been developed for species level identification. Even with the use of sophisticated biochemical characterization, some species can only be identified on the basis of DNA or rRNA sequence analysis, or DNA-DNA hybridization studies. This means that only a handful of laboratories are able to identify unusual *Legionella* spp., especially novel ones.

### Growth Rate and Colonial Morphology

When plated from primary clinical or environmental specimens, colonies of legionellae appear on selective or nonselective BCYE $\alpha$  agar within 3–14 days (average 3–4 days) of incubation. Growth from a fresh colony generally requires

18–36 h of incubation, depending on the density of the inoculum; a heavy inoculum usually grows overnight, and a light inoculum may take several days to grow on subculture. Some of the more fastidious species, such as *L. oakridgensis*, *L. sainthelensi* and *L. rubrilucens* may require up to several more days of incubation for growth to appear on subculture; these and some other *Legionella* spp. may grow better in the presence of 3–5% CO<sub>2</sub>. Recognition and preliminary identification of the bacteria are facilitated by the use of a dissecting microscope for the examination of the media. *Legionella* colonies display a characteristic appearance resembling "ground glass," or an opal, when viewed under obliquely transmitted light. Very young colonies are gray, flat, round, entire and about 0.25–0.5 mm in diameter; at this stage they are very difficult to see with the naked eye. Within a day the colonies become raised, round, entire and convex, and are 1–2 mm in diameter. The colony edge usually displays a birefringent band, which is either greenish blue or pink depending on the species; this coloration may be pleomorphic but does not breed true. Up to this stage, the colonial morphology is distinctive for experienced observers. As the colonies become several days older, the ground-glass appearance and the birefringent edge tend to disappear and the colonies become umbonate and sometimes tuberculated; the maximum diameter is usually about 3–4 mm. Eventually the colonies flatten completely. Very old colonies are impossible to distinguish with certainty from other bacteria, even for experts. This makes it especially important to examine plates for early growth when plating from primary specimens, or when contamination with other bacteria is a possibility. When the colonies are illuminated with a Woods' lamp the colonies of most species will exhibit a dull yellow fluorescence (Table 3). The colonies of *L. bozemanii*, *L. gormanii*, *L. dumoffii* and several other species, display a very striking blue-white autofluorescence under similar conditions of illumination, whereas colonies of *L. rubrilucens*, *L. erythra* and some strains of *L. taurinensis* produce a red fluorescence.

Most *Legionella* spp. growing on BCYE $\alpha$  medium have a characteristic musty smell. This odor is specific enough to aid in the identification of the bacterium. Incubators containing several plates with heavy growth of *Legionella* spp. emit this odor when first opened, as do individual culture plates.

### Cellular Morphology and Biochemical Characteristics

The cellular morphology of *Legionella* spp. bacteria is dependent on growth phase, both in vitro

Table 3. Selected characteristics of the *Legionella* spp.

<i>Legionella</i> spp.	Fluorescence <sup>a</sup>	TYE browning	Oxidase	Catalase	Gelatinase	β-Lactamase	Hippurate hydrolysis	Major cellular fatty acid(s) <sup>b</sup>
<i>adelaidensis</i>	NC	–	–	+	+	–	–	16:1, 16:0
<i>anisa</i>	bw/ylg	+	+	+	+	+	–	a15:0
<i>birminghamensis</i>	YG	–	–	+	+	+	–	a15:0
<i>bozemanii</i>	BW	+	+	+	+	+	–	a15:0
<i>brunensis</i>	NC	+/-	+/-	+	+	+	–	a15:0, a17:0
<i>cherrii</i>	BW	+	+	+	+	+	–	i16:0, 16:1
<i>cincinnatiensis</i>	YG/NC	+	+	+	+	+	–	i16:0, cyc-17:0
<i>dumoffii</i>	BW	+/-	+/-	+/-	+	+	–	a15:0
<i>erythra</i>	R	+	+	+	+	+	–	16:0, 16:1
<i>fairfieldensis</i>	NC	–	–	+	–	–	–	i16:0, 16:1; variable
<i>feelei</i>	NC	–	+/-	+	–	–	+/-	a15:0, i16:0, 16:1
<i>geestiana</i>	NC	+/-	+/-	+	+	+	+/-	16:1, 16:0
<i>gormanii</i>	BW	+	+	+	+	+	–	a15:0
<i>gratiana</i>	NC	–	–	+	+	+	–	i16:0, 16:0, 16:1
<i>hackeliae</i>	YG	+	+	+	+	+	–	a15:0
<i>israelensis</i>	NC	–	–	+	+/-	+	–	a15:0, i16:0
<i>jamestownensis</i>	YG	+	+	+	+	+	–	a15:0
<i>jordanis</i>	YG	+	+	+	+	+	–	a15:0
<i>lansingensis</i>	NC	–	–	+	–	–	–	a17:0, a15:0
<i>londiniensis</i>	NC	+	+	+	+	+	+/-	a17:0, 16:1, 16:0
<i>longbeachae</i>	YG	+	+/-	+	+	+/-	–	i16:0, 16:1
<i>“lytica”</i>	NA	NA	NA	NA	NA	NA	NA	NA
<i>maceachernii</i>	YG	+	+	+	+	–	–	a15:0
<i>micdadei</i>	YG	–	+/-	+	–	–	–	a15:0
<i>moravica</i>	NC	+/-	–	+	+	+	–	16:1, 16:0
<i>nautarum</i>	NC	–	+	+	–	+	–	16:1, 16:0
<i>oakridgensis</i>	YG	+	–	+	+	+/-	–	i16:0, 16:1
<i>parsiensis</i>	BW	+	+	+	+	+	–	a15:0
<i>pneumophila</i>	YG	+	+/-	+/-	+	+	+	i16:0, 16:1
<i>quateirensis</i>	NC	+	–	+	+	+	–	16:1, i16:0
<i>quinlivanii</i>	YG	+	–	+	+	–	–	a15:0, a17:0
<i>rubrilucens</i>	R	+	–	+	+	+	–	i16:0, 16:1
<i>sainthelensi</i>	YG	+	+	+	+	+	–	i16:0, 16:1
<i>sainticrucis</i>	YG	+	+	+	+	+	–	i16:0, 16:1
<i>shakespearei</i>	NC	–	+/-	+	+	+	–	i16:0, 16:1
<i>spiritensis</i>	YG	+	+/-	+	+	+	+	i16:0, 16:1
<i>steigerwaltii</i>	BW	+	–	+	+	+	–	a15:0
<i>taurinenis</i>	R/ylg	–(96%)/+	+/-	+	+	+	+/-	i16:0, a15:0
<i>tucsonensis</i>	BW	–	–	+	+	+	–	a15:0, 16:1
<i>wadsworthii</i>	YG	–	–	+	+	+	–	a15:0
<i>waltersii</i>	NC	–	+	+	+	+	+	16:1, i16:0
<i>worsleiensis</i>	NC	+	–	–	+	+	–	16:1, 16:0

Symbols: –, negative; +, positive; and +/-, most strains positive, or all strains weakly positive.

Abbreviations: TYE, tyrosine-containing buffered yeast-extract medium without charcoal; and NA, not applicable.

<sup>a</sup>Colony color when illuminated with long wave UV light: BW, blue-white; YG, yellow-green; NC, no distinct color; R, red; and R/ylg, majority are red, some are yellow-green. This color is dependent on growth conditions and colony age, and may be lost with serial passage. “No autofluorescence” reported by some authors is signified by “NC”; it is unclear whether these colonies fluoresce a yellow-green color.

<sup>b</sup>These results may differ according to growth conditions and methods of analysis. The fatty acids are listed in order of predominance, with only the first two to three predominant fatty acids listed.

<sup>c</sup>Listed in order of predominance when not in numerical order; otherwise species designated as possessing multiple major ubiquinones containing them in approximately equal amounts.

<sup>d</sup>These strains may grow on BCYE $\alpha$  media made without L-cysteine, but generally only after serial passage. L-Cysteine is required for primary isolation for all of these species, and for optimal growth for some.

Data compiled from original published descriptions of the species, from unpublished data (P. Edelstein), and from Gilbert (1985).

and in vivo. The bacterium can vary from highly motile tiny (1  $\mu$ m) coccoid forms during the last stages of intra-amoebal growth to very long and nonmotile filaments (2–3  $\times$  20  $\mu$ m) during stationary phase on solid media. Bacteria grown to

early log phase are nonmotile short bacilli, in broth, on solid media, and in the early stages of intra-amoebal and intra-macrophage growth. In late stationary phase on solid media, and in broth, there can be a mixture of filamentous

forms, medium-length rods, and short motile coccoid forms (Rowbotham, 1986; Anand et al., 1983; Byrne and Swanson, 1998; Pine et al., 1979).

Ultrastructural studies of the bacteria show an arrangement characteristic of Gram-negative rods, with typical trilaminar inner and outer membranes (Chandler et al., 1979b; Neblett et al., 1979; Rodgers, 1979; Watson and Sun, 1981; Rodgers and Davey, 1982). A definite peptidoglycan structure also is seen in the cell wall (Flesher et al., 1979; Rodgers and Davey, 1982). *Legionella micdadei* has a unique electron dense layer in the periplasmic space that is not seen in *L. pneumophila* (Gress et al., 1980; Hébert et al., 1984). Several species, except *L. micdadei*, appear to have an extracellular polysaccharide capsule or slime layer that can be visualized by ruthenium red staining, even though the bacteria appear unencapsulated by other means (Hébert et al., 1984). Both flagellae and pili have been observed in some ultrastructure studies (Chandler et al., 1980; Rodgers et al., 1980b).

Staining characteristics depend to some degree on the source of the bacteria. Typically, Gram stain does not reliably stain the legionellae in clinical specimens, although bacteria cells from artificial media stain somewhat better. The substitution of basic fuchsin for the safranin counterstain in the Gram technique makes the legionellae more readily visible. Staining of the legionellae can also be accomplished using crystal violet and methylene blue, although these methods are not currently used (de Freitas et al., 1979; Pasculle et al., 1980). *Legionella micdadei* is unusual in that the cells are weakly acid fast when present in tissue but readily lose this property after a single passage on artificial media. The reason for this is unknown (Pasculle et al., 1980). *Legionella pneumophila* has also been reported to be positive on acid-fast staining of bronchoalveolar lavage material (Bentz et al., 2000). The legionellae can also be readily stained by the Gimenez stain (McDade et al., 1977) and the Dieterle silver (Chandler et al., 1977) stains, but neither technique is specific for these bacteria.

The legionellae are chemoorganotrophic do not possess a glucose transport system, nor do they ferment or oxidize other carbohydrates. Early studies using several defined media suggested that methionine, arginine, threonine, serine, isoleucine, leucine and valine are required by the legionellae, in addition to cysteine. Serine, glutamate and perhaps threonine appear to serve as the primary carbon and energy sources for the legionellae and are catabolized via the Krebs cycle. Carbohydrate synthesis occurs through gluconeogenesis via the Embden-Meyerhof pathway (Pine et al., 1979; George et al., 1980; Tesh and Miller, 1981; Tesh and Miller, 1983a;

Tesh et al., 1983b).  $\alpha$ -Ketoglutaric acid is a growth stimulant and a component of most growth media for the bacterium (Pine et al., 1986; Edelstein, 1981).

Members of the genus *Legionella* cannot be identified with any degree of certainty using traditional biochemical testing. This is because of the general biochemical inertness of the bacterium when using conventional biochemical tests, and because many of the species can only be correctly identified using molecular methods. An extensive panel of biochemical tests has been shown to be useful in identification, but these methods have not been widely used or validated after their initial description, nor has their use been described with newly recognized species (Vesey et al., 1988). The tests that appear to be most useful for bacterial identification include demonstration of L-cysteine growth dependence, serotyping to detect *L. pneumophila* and *L. pneumophila* serogroup 1, determination of cellular fatty acids and ubiquinones, and determination of the macrophage infectivity potentiator (mip) gene sequence (Ratcliff et al., 1998). The few phenotypic tests that can be used to separate these organisms into groups are given in Table 3. All species are motile via one to three polar or subpolar flagella and the flagella of all legionella species appear to be antigenically identical (Bornstein et al., 1991). Only *L. oakridgensis* appears to be nonmotile. Determination of flagellation may be difficult as flagellar production is growth-condition dependent (Heuner et al., 1999). It is much easier to demonstrate flagellae in plate-grown than broth-grown bacteria, especially from plates that have been incubated at room temperature. The Ryu stain is a convenient method for staining the flagellae (Kodaka et al., 1982; Edelstein, 1985b). Tests for nitrate reductase and urease are negative for all species. Most strains are reported to be catalase positive when whole cells are tested for the ability to decompose hydrogen peroxide ( $H_2O_2$ ); the catalase reaction can be very weak. Methods that enhance the sensitivity of the catalase reaction include performance of the test using capillary tubes and the use of 3%  $H_2O_2$  in 10% Tween 80 (Edelstein, 1985b). Studies using cell-free extracts, however, have demonstrated the *L. pneumophila* and *L. gormanii* have only peroxidase activity, whereas other *Legionella* species in actuality do possess catalase (Pine et al., 1984). Most species liquefy gelatin, using an assay medium in which gelatin replaces agar in the formulation of BCYE $\alpha$  medium (Edelstein, 1985b). *Legionella pneumophila* and a few other species hydrolyze hippurate (Edelstein, 1985b). Most strains produce a  $\beta$ -lactamase that is active against cephalosporins and can readily be demonstrated using nitrocefin (Marre et al., 1982).

Browning of tyrosine-containing medium is also a helpful phenotypic test, using BCYE $\alpha$  medium made without charcoal and containing tyrosine (Edelstein, 1985b).

## Cellular Lipids, Quinones and Carbohydrates

The cell wall of the legionellae contains large amounts of branched-chain fatty acids and only minor amounts of hydroxy acids (Mayberry, 1981; Lambert and Moss, 1989; Table 3). Accurate cellular fatty acid composition determination requires the use of a high quality gas liquid chromatograph and capillary columns, and also may require the use of mass spectroscopy. Fatty acid characterization is too complex to be used in the routine identification of *Legionella* spp. isolates, but can be of great help to taxonomists, or to identify unusual isolates. Analyses for very long chain fatty acids and unusual lipopolysaccharides also can be used to help identify some *Legionella* spp. (Moll et al., 1992; Sonesson and Jantzen, 1992; Sonesson et al., 1989; 1993; 1994a; 1994b; 1994c; Jantzen et al., 1993). Characterization of complex cellular sugars also can be used to characterize and identify some *Legionella* spp. (Fox et al., 1990). The instrumentation for fatty acid and polysaccharide analysis is very expensive and difficult to maintain, and requires a high level of technical expertise for its use. In addition, extraction of the fatty acids is labor intensive and utilizes hazardous chemicals, although a relatively rapid technique has been described (Nalik et al., 1992).

The legionellae also are unusual in that their isoprenoid quinones are ubiquinones with side chains containing greater than nine isoprenoid units (Karr et al., 1982; Moss and Guerrant, 1983; Marmet et al., 1988; Lambert and Moss, 1989). The determination of fatty acid and ubiquinone profiles is generally too complex for routine laboratory use. These procedures are, however, very helpful in the preliminary characterization of isolates by reference laboratories.

## Identification Techniques

### L-Cysteine Growth Dependence

Establishing the L-cysteine growth dependence (characteristic of the genus *Legionella*; Benson and Fields, 1998) is usually the first step for a Gram-negative bacterium growing on BCYE $\alpha$  medium with colonial morphology typical of *Legionella* spp. In most laboratories, this is done by inoculating the isolate onto a cysteine-deficient BCYE $\alpha$  agar plate (Isenberg, 1979).

Other cysteine-deficient media (such as sheep blood agar) may be used for this purpose, but occasional strains of other heterotrophic environmental bacteria may be encountered that will grow on BCYE $\alpha$  and not on blood agar (Thacker et al., 1981). Placement of an L-cysteine-containing disk on cysteine-deficient BCYE $\alpha$ , or on tryptic soy blood agar, will result in growth of the *Legionella* bacteria around the disk (Smith, 1982). In addition, *Brucella* blood agar will support the growth of at least some *Legionella* bacteria, as will several other rich media (P. Edelstein, unpublished observation). After serial passage, several *Legionella* species can grow on media without L-cysteine, but generally still exhibit L-cysteine dependence on the first or second passage on artificial media (Table 3).

### Serotyping

Several of the various recognized species and serovars of *Legionella* can be identified by serological methods. Fluorescein-conjugated antisera for direct immunofluorescence testing can be used for the rapid presumptive identification of isolates (Cherry et al., 1978; Wilkinson, 1987a; Edelstein and Edelstein, 1989a). Because *L. pneumophila*, and especially *L. pneumophila* serogroup 1, accounts for the preponderance of clinical isolates (Reingold et al., 1984; Dournon et al., 1985; Marston et al., 1994), most clinical isolates can be identified using commercially available antisera to *L. pneumophila* serogroup 1, and to *L. pneumophila*. A monoclonal antibody conjugate that reacts with all known serogroups of *L. pneumophila* is commercially available (<http://www.biorad.com>[Biorad] [formerly Genetic Systems] Hercules, CA; Edelstein et al., 1985d; Tenover et al., 1986; Gosting et al., 1984). Several firms make *L. pneumophila* serogroup 1 specific antibodies, as well as antibodies for a number of other species and serogroups m-TECH (<http://www.4m-tech.com>); MarDx, now Trinity Biotech (<http://www.trinitybiotech.com/prod.htm>); Scimedex (<http://www.globaldx.com/products.html>).

Tests need to be interpreted with caution. A prozone phenomenon can cause a false-negative reaction if the bacterial suspension being tested is too heavy. In addition, the monoclonal antibody reacts with a bacterial antigen that is hidden by prolonged Formalin fixation (Gosting et al., 1984), making adherence to the manufacturer's guidelines important.

Simple slide agglutination tests for the identification of the legionellae are also commercially available, although their performance characteristics have not been described Oxoid (<http://www.oxidshop.com>); Mast (<http://www.mastgrp.com/intro.htm>).

Extensive crossreactions among the legionellae requires that the polyclonal antisera used for this procedure and for immunofluorescence be subjected to reciprocal adsorption to remove crossreacting antibodies. Such cross-absorbed antisera are not commercially available, making most identification by serotyping subject to error. The monoclonal antibody to *L. pneumophila* does not crossreact with other *Legionella* spp, nor does the polyclonal antibody to *L. pneumophila* serogroup 1. However, there are several *L. pneumophila* serovars that share antigens with different serogroups, such as *L. pneumophila* "serogroup 1,4" and *L. pneumophila* "serogroup 1,5." Within *L. pneumophila* serogroup 1, there may be a wide range of reactivity between antisera made to the Philadelphia 1 and Bellingham strains, such that antibodies to one of these two polar serovars may not react with the other serovar. Several novel *Legionella* species have been identified that react with antibody to other species, making molecular identification crucial for definitive identification.

### Molecular Methods for Species Identification

DNA-DNA hybridization at different temperatures is the definitive means of identifying currently recognized *Legionella* spp., and of differentiating them from novel species (Brenner et al., 1979; Brenner, 1987; Benson and Fields, 1998). A commercial test kit, manufactured and sold in Japan (Kyokuto Seiyaku Co; "DDH-Legionella"), takes advantage of this technology; no published evaluations of the performance of the kit are available. Evaluations of a research kit showed excellent performance (Ezaki et al., 1990; Hatanaka et al., 1992), and evaluation of the underlying methodology shows excellent correspondence with the standard method (Goris et al., 1998). The most promising new method appears to be sequencing of the *mip* gene, using degenerate primers, as this can be accomplished by laboratories with access to thermal cyclers and automated sequencing instruments (Ratcliff et al., 1997; 1998). Other methods that have been tested include 16S and 5S rRNA sequencing, as well as random amplified polymorphic DNA analysis (RAPD; Ludwig and Stackebrandt, 1983; MacDonell and Colwell, 1987; Böttger, 1989; Fry et al., 1991; Hookey et al., 1996; Robinson et al., 1996; Pinar et al., 1997; Riffard et al., 1998a; Bansal and McDonell, 1997; LoPresti et al., 1998b). Sequencing of 5S rRNA appears to be unable to discriminate between all species (Murdoch et al., 1999b), whereas sequencing of 16S rRNA may be more discriminatory (Riffard et al., 1998a; Benson and Fields,

1998). A thorough comparison of the use of RAPD versus that of other molecular techniques has not been reported.

## Laboratory Diagnosis

### Nonspecific Findings

A number of nonspecific laboratory abnormalities may occur in Legionnaires' disease. These are abnormal urinalysis with proteinuria and hyaline or granular casts; hypophosphatemia, hyponatremia, and less commonly, elevations of aldolase or creatine kinase (Arrizabalaga et al., 1984; Cheung, 1980; Heule et al., 1986; Tsai et al., 1979). Extreme electrolyte abnormalities related to massive diarrhea occur rarely (Foltzer and Reese, 1985). Renal failure has been reported rarely. The renal failure may occur with myositis and/or marked elevations of creatine kinase and aldolase or with myoglobinuria and may represent rhabdomyolysis (Lin et al., 1995; Cases et al., 1987; Fenves, 1985; Hall et al., 1983; Poulter et al., 1981; Harvey et al., 1980; Williams et al., 1980). In addition, interstitial nephritis, mesangial proliferative and progressive glomerulonephritis all have been reported (Pai et al., 1996; Hariparsad et al., 1985; Wegmüller et al., 1985). The white blood cell count is elevated (>10,000/ $\mu$ l), often with a left shift in about one-half to three-fourths of patients. Leukopenia and thrombocytopenia are observed in severe disease. Serum cold agglutinins and even cold agglutinin disease also have been observed in several cases (King and May, 1980). Disseminated intravascular coagulation is observed rarely, usually in the setting of severe respiratory failure and diffuse lung infiltrates (Dowling et al., 1983; Gregory et al., 1979). Elevations of lactic dehydrogenase, alkaline phosphatase and aspartate aminotransferase are also common (Kirby et al., 1980; Cunha, 1987). Bilirubin elevation is less common, but there are several case reports of jaundice as a presenting sign of illness, as well as severe jaundice occurring as part of the disease (Verneau et al., 1987; García et al., 1991; Levin et al., 1993). Hypoxemia is usually in proportion to the degree of pulmonary involvement seen on radiographs (Kirby et al., 1980; Cunha, 1987). None of the nonspecific laboratory findings predicts with reasonable certainty whether a patient has Legionnaires' disease or another cause of pneumonia (Edelstein, 1993a; Fang et al., 1990a).

### Specific Findings

There are four currently used standardized methods for the specific laboratory diagnosis of



*Legionella* infections (Table 2). These are determination of antibody level, demonstration of the bacterium in tissues or body fluids by using immunofluorescent microscopy, actual isolation of the organism on culture media, and detection of antigenuria (Aguero-Rosenfeld and Edelstein, 1988; Edelstein, 1987a; Plouffe et al., 1995; Ramirez and Summersgill, 1994; Birtles et al., 1990). Detection of bacterial DNA using PCR is being used experimentally, but has yet to be widely adopted (Helbig et al., 1999; Murdoch et al., 1996; Murdoch et al., 1999a; Weir et al., 1998; Koide and Saito, 1995; Maiwald et al., 1995; Jaulhac et al., 1992; Jaulhac et al., 1998). A commercial DNA probe test used to diagnose *L. pneumophila* rRNA is no longer being produced (Edelstein et al., 1987c; Doebbeling et al., 1988; Pasculle et al., 1989).

### Antibody Estimation

Estimation of serum antibody to *L. pneumophila* is a commonly used means of diagnosing Legionnaires' disease (Edelstein, 1997). The vast majority of laboratories use an indirect immunofluorescent microscopy (IFA) technique to determine antibody concentrations. Only measurement of antibody to *L. pneumophila* serogroup 1 by IFA is well standardized, and is the "gold standard" test used to diagnose Legionnaires' disease by serologic means. Several commercial enzyme immunoassays (ELISA) can be successfully used to screen antibodies for elevated levels, which can then be confirmed with the *L. pneumophila* serogroup 1 IFA (Harrison et al., 1999).

There are two widely used reference methods of antigen preparation for the IFA test: heat fixation of plate-grown bacteria and formalin fixation of chicken embryo yolk-sac grown bacteria (Edelstein, 1997). The latter method may be more specific although large head-to-head comparative studies have not been performed (Harrison and Taylor, 1982). Many commercial laboratories sell kits containing Formalin-fixed plate-grown bacteria, but these kits are not known to provide the same results as either of the reference methods. Use and results interpretation of these commercial IFA kits may not give results similar to those obtained using the reference methods.

About three-quarters of patients with culture-proven Legionnaires' disease caused by *L. pneumophila* serogroup 1 develop a fourfold rise in IFA titer from one to nine weeks after onset of illness. The mean time required for demonstration of seroconversion is about two weeks; however, up to 25% of seroconversions are missed unless serum is collected up to eight weeks after onset of illness. Because 5–30% of healthy pop-

ulations sampled have *L. pneumophila* serogroup 1 antibody titers of 1 : 128 or greater when using a heat-fixed antigen, only a fourfold rise in titer to 1 : 128 or greater can be considered significant; significant titers for formalin-fixed antigen are a rise in titer to 1 : 32 or greater. In the face of an outbreak of Legionnaires' disease, a single titer of >1 : 256 (heat-fixed antigen) in a patient with a compatible clinical illness has been considered significant. However, in the sporadic case such single high titers cannot be interpreted (Plouffe et al., 1995). The specificity of the IFA test in a hospitalized population is not well known; this probably approximates 90% for a fourfold titer rise, although in an epidemic situation in nonhospitalized patients the specificity is close to 100%.

Crossreactions for the IFA test have been reported in patients with tuberculosis, pneumococcal pneumonia, pseudomonas pneumonia, exacerbations of cystic fibrosis, tularemia, plague, *Bacteroides fragilis* bacteremia, and leptospirosis (Bornstein et al., 1987; Edelstein et al., 1980a). Up to 20% of patients with campylobacter enteritis have been reported to have crossreactive antibody to *L. pneumophila* (Boswell, 1996; Marshall et al., 1994; Boswell and Kudesia, 1992). Antibody testing is most specific when using *L. pneumophila* serogroup 1 antigen, especially when a Formalin-fixed antigen is used. The specificity of a fourfold antibody rise to the serogroup 1 antigen is at least 99%, whereas test specificity is considerably lower when using antigens from other *L. pneumophila* serogroups, or from other species. Aside from variability in antibody test results due to the type and numbers of antigens tested, other methodologic variables may affect results. None of the commercially available kits for IFA determination of antibody status use exactly the same methods that were used for determination of test performance. In addition there has never been conducted a valid study of the clinical specificity and sensitivity of IFA kits using multiple antigens, or antigens other than *L. pneumophila* serogroup 1. Thus it is improper to use cutoff values determined for *L. pneumophila* serogroup 1 antibodies when interpreting the meaning of antibodies to other antigens. Because of this, it is better not to test for antibody to antigens other than *L. pneumophila* serogroup 1, except for epidemiological studies. In addition to the possibility of crossreactions and the fact that serologic testing is retrospective in nature (and does not influence choice of therapy), the other major drawback of diagnosing *Legionella* infections using serologic means is that the test may be negative because the serotype of the infecting organism is not tested for. Thus, serologic testing in the diagnosis of this disease is much more helpful to epidemi-

ologists than to clinicians caring for individual patients.

Several EIA kits that measure *L. pneumophila* antibodies are commercially available, and are manufactured or sold in the United States by Wampole Laboratories, Zeus Diagnostics, and Sigma Diagnostics. The Sigma kit is made by Zeus Diagnostics and is essentially the same kit. Unfortunately there is only one published evaluation of some of these kits (Harrison et al., 1999), and one publication describing the performance of a commercial kit that is not widely available (Nguyen et al., 1995). All of these kits screen for IgA, IgM and IgG antibodies to *L. pneumophila* serogroups 1 to 6. The sensitivity of the kits for measurement of antibody to *L. pneumophila* serogroups 2 to 6 is unknown, but they do appear to be reasonably sensitive (70–90%) for the estimation of serogroup 1 antibodies. Test specificities are about 98%. The tests are designed to detect relatively high IFA titer antibody, in the range of 1 : 128 to 1 : 256. Because the antigens detected are a pool of serogroups, a positive enzyme immunoassay (EIA) result can be due to cumulative low-level antibodies to different serogroups, as has been observed for the IFA assay using multiple antigens. Because of this, the positive predictive accuracy of the Wampole assay is about 90%, in other words about 90% of EIA-positive or indeterminate sera will have IFA titers (heat-killed antigen)  $\geq 64$ , with most  $>64$  (P. Edelstein, unpublished data). All positive EIA tests should be confirmed by use of the IFA test using a *L. pneumophila* serogroup 1 antigen (Harrison et al., 1999). The best use of the EIA test is as a screening assay of convalescent-phase sera. Any such sera yielding positive or indeterminate results can then be tested in parallel with the acute-phase serum by IFA. Using this scheme, about 10% of serum pairs so selected are positive for a significant change in IFA titer (P. Edelstein, unpublished data).

An evaluation of the optimal characteristics for antibody detection by ELISA show that a sonicated antigen used with IgA detection was most sensitive, whereas use of a lipopolysaccharide antigen with IgG and IgM detection, was most specific (Bangsberg et al., 1994). No commercial ELISA assay utilizes LPS antigens.

### Detection of Bacterial Antigen in Tissues

Immunofluorescent microscopy of respiratory tract secretions, lung and pleural fluid is one of the rapid test methods available to establish a laboratory diagnosis of Legionnaires' disease. When this technique is used with an antibody conjugated with a fluorochrome, it is termed the "direct immunofluorescence assay" (DFA). About 2–3 hours is required to complete this

test. This technique has been used very successfully with expectorated sputum, endotracheal suction aspirates, lung biopsies and transtracheal aspirates. Use of secretions or biopsies obtained by bronchoscopy has not resulted in high yield in our experience, although others have had more success. Pleural fluid examination in patients with Legionnaires' disease usually is unproductive, both in terms of culture and DFA positivity, but it has occasionally been helpful. The true sensitivity of the DFA test is unknown. About 25–70% of patients with culture-proven Legionnaires' disease have positive sputum DFA tests for *L. pneumophila*; test specificity is greater than 99.9%; therefore, a negative result does not rule out disease and a positive result is almost always diagnostic of it (Edelstein et al., 1980b). Antisera used to detect infection caused by *L. pneumophila*, especially *L. pneumophila* serogroups 1 to 4, are more specific than other antisera; use of antibodies other than these is problematic because of crossreactions, and should be confined to testing normally sterile tissues and fluids (e.g., lung biopsies), if at all possible. A commercially available (from <http://www.biorad.com>{Biorad} [formerly Genetic Systems], Hercules, CA) monoclonal antibody DFA reagent which reacts with all serogroups of *L. pneumophila* provides optimal specificity and eliminates the need to use multiple antisera to detect this species (Edelstein et al., 1985d; Tenover et al., 1986). Unlike polyvalent reagents, it does not crossreact with some *Pseudomonas*, *Flavobacterium-Xanthomonas* and *Bacteroides* strains; however it cannot be used to stain tissues fixed in formalin for prolonged periods. When sera other than the monoclonal antiserum are used, it is wise to interpret with extreme caution positive smears of tissues or fluids that might contain large quantities of known crossreacting bacteria. Exceptional skill is needed to read DFA test slides properly. DFA tests of sputum remain positive for 2–4 days after the initiation of specific antibiotic therapy for Legionnaires' disease, and often much longer in cases of cavitary pulmonary disease.

### Culture Isolation from Clinical Specimens

Isolation of *Legionella* from clinical specimens is routinely performed in many laboratories. The medium used, supplemented charcoal yeast extract medium (BCYE $\alpha$ ), is easily prepared by any large clinical microbiology laboratory and can be made in a selective form. Use of selective media and specimen decontamination with acid are obligatory for optimal culture yield from normally nonsterile tissues and fluids. To obtain optimal yield, specimens with and without acid pretreatment are plated on three different media

(all commercially available; total of six plates): BCYE $\alpha$  (nonselective), BMPA (selective, also called "CAP" or "PAC"), and MYEA (selective, also called "PAV" or "VAP"; Edelstein, 1985a; 1985b; 1987a; Vickers et al., 1987). Use of two different selective media is required as some *Legionella* spp., and some strains of *L. pneumophila* serogroup 1, will not grow on BMPA, which is the most selective medium. Use of multiple media also increases the chances of detecting very small numbers of *Legionella* spp. bacteria present in the specimen. Specimen dilution before plating is also important, as *Legionella* spp. growth may be inhibited by certain cations, other bacteria and by tissue factors. The organism has been successfully isolated from sputum, transtracheal aspirates, endotracheal suction specimens, blood, lung biopsy, pleural fluid, bronchial lavage, pericardial fluid, peritoneal fluid, wounds, bowel abscesses, prosthetic heart valves, brain abscesses, myocardium, kidney, liver, vascular grafts and respiratory sinuses. Cultures generally remain positive for several days after the initiation of antimicrobial therapy, and may remain positive for weeks or months from pulmonary abscesses. Broad-spectrum antimicrobial therapy decreases culture yield.

Like the sensitivity of all other tests for Legionnaires' disease, that of culture is unknown. Nevertheless, it is often more sensitive than other available diagnostic tests. Only about 75% of people with culture-proven Legionnaires' disease develop antibody conversion; less than half have positive sputum DFAs; and a large, although poorly defined, fraction of all those without *L. pneumophila* serogroup 1 infections have negative urinary antigen tests. However, it is common in community-based outbreaks of Legionnaires' disease for sputum cultures to be negative, while urinary antigen tests, and somewhat less commonly, serum antibody tests are positive. It is unclear how often the negative sputum cultures in this situation are due to inexpert laboratory testing, improper or inadequate sputum collection, or both. It is likely that urinary antigen testing is the most sensitive test for Legionnaires' disease in patients with *L. pneumophila* serogroup 1 disease.

Once isolated on a culture medium, *Legionella* spp. are relatively easy to identify to the genus level. This is based on characteristic colonial morphology, growth requirement for L-cysteine, and serotyping. Also helpful are characteristic branched-chain cellular fatty acid and ubiquinone compositions, and lack of acid production from carbohydrates. Because of the antigenic complexity of Legionellae, it is impossible to serologically distinguish several of the species. Combined with a paucity of other useful phenotypic characteristics, this means that identifica-

tion to the species level can be very difficult, except for extremely sophisticated research laboratories. However, all laboratories should be able to identify *L. pneumophila*, *L. pneumophila* serogroup 1, and other Legionellae to the genus level. This is because of the availability of a *L. pneumophila* species-specific monoclonal antibody (Genetic Systems), of many available and reliable *L. pneumophila* serogroup 1 polyclonal antibodies, and because of the specificity of colonial morphology, Gram stain appearance, and L-cysteine dependence for all *Legionella* species (see Table 3 for exceptions).

Microscopic morphology of the organisms depends on growth conditions. They are usually very faintly staining small coccobacilli in lung and sputum, and often long and filamentous bacilli when taken from a culture plate. Although colonies growing on artificial media stain well with Gram stain (especially if basic fuchsin rather than safranin is used as the counterstain), the bacteria are exceptionally difficult to visualize using this stain on fixed lung specimens, and often with fresh tissues and fluids also. For tissue examination, the Giménez stain is more effective, much simpler and more sensitive than the Dieterle silver impregnation stain (Edelstein et al., 1980b).

### Polymerase Chain Reaction

Although PCR has been used to diagnose Legionnaires' disease, this test is still regarded as a research tool. In most studies the sensitivity of PCR has been lower than that of culture. *Legionella* spp. DNA has been successfully detected with PCR in human and experimental animal urine, although the urine PCR may not be as sensitive as the urine antigen test. Urine PCR may be useful for detecting *L. pneumophila* serogroups and species not detected by the antigen test (Helbig et al., 1999; Murdoch et al., 1996; Murdoch et al., 1999a; Weir et al., 1998; Koide and Saito, 1995; Maiwald et al., 1995; Jaulhac et al., 1992; Jaulhac et al., 1998).

### Antigenuria

*Legionella pneumophila* serogroup 1 antigenuria can be detected by using an enzyme immunoassay or radioimmunoassay (Tilton, 1979; Kohler et al., 1981; 1984; Sathapatayavongs et al., 1982). There are several commercial enzyme immunoassay kits available; the best studied one is made by the Binax Company, and was formerly made by Dupont (Aguero-Rosenfeld and Edelstein, 1988; Plouffe et al., 1995; Hackman et al., 1996; Kazandjian et al., 1997; Domínguez et al., 1998). It is now made by Binax and sold by (<http://www.wampolelabs.com/>

[in\\_fp\\_Legionella.htm](#){Wampole Labs}, Cranbury, NJ).

The sensitivity of the Binax/Wampole assay is quite high; more than 95% of urines collected from patients with culture-proven *L. pneumophila* serogroup 1 disease are positive with the test. About 80% of urines collected with patients with only serologically proven Legionnaires' disease are positive in the assay; inasmuch as some of these patients may have had infection caused by other *L. pneumophila* serogroups, other *Legionella* spp., or may not have had Legionnaires' disease (but only a false-positive antibody test), the sensitivity of the assay is probably higher than 80% (Kohler, 1986). The clinical sensitivity yield of urinary antigen testing has uniformly been much higher than culture in outbreaks of Legionnaires' disease, and use of this test is essential when investigating epidemics of *L. pneumophila* serogroup 1 pneumonia (Jernigan et al., 1996; Brown et al., 1999; Fiore et al., 1998). In sporadic cases of community-acquired Legionnaires' disease, the sensitivity of antigenuria detection of *L. pneumophila* can also be higher than that of sputum culture, even when excellent culture techniques are used (P. Edelstein, unpublished observation); in laboratories not utilizing optimal culture techniques the disparity in diagnostic yield for the two test types can be dramatic. Patients with severe Legionnaires' disease, such as those with respiratory failure and multilobar pneumonia, may excrete bacterial antigen in their urine for weeks to months after recovery from pneumonia; this phenomenon does not occur in patients with mild Legionnaires' disease (Kohler et al., 1984). Test sensitivity can be enhanced by urine concentration before testing (Domínguez et al., 1996; 1997).

The Binax/Wampole assay is largely specific for *L. pneumophila* serogroup 1 and has low sensitivity for detection of antigenuria caused by infections with other *L. pneumophila* serogroups or other *Legionella* spp. Some crossreactions do occur, so a positive urinary antigen test does not exclude infection caused by other *L. pneumophila* serogroups (Kohler et al., 1985). Crossreactions can be demonstrated with a number of *L. pneumophila* serogroups using culture extracts (Domínguez et al., 1998). False-positive tests are often associated with rheumatoid-like factors, which can be eliminated by boiling the sample before testing; the *Legionella* antigen itself is heat stable (Kohler et al., 1981). In addition, cloudy urines rarely produce false-positive tests, although when they do, the false positivity can be eliminated by pre-test centrifugation.

The Biotest urinary antigen detection assay ([http://www.biotest.com](#){Biotest AG}, Dreieich, Germany) claims reactivity with a variety of *L. pneumophila* serogroups. However, there is little

reliable clinical data to evaluate this possible difference; an in vitro study of several different *L. pneumophila* antigens showed no significant differences between the two tests (Domínguez et al., 1998). In the same study, there was no significant difference in the ability to detect *L. pneumophila* serogroup 1 antigenuria. ([http://www.intracel.com/bartels/bartels.html](#){Bartels Diagnostics}) has recently introduced a *L. pneumophila* serogroup 1 antigen assay, but only abstracts and an ([http://www.fda.gov/cdrh/pdf/k991074.pdf](#){FDA summary}) of evaluations are available.

Recently, urinary antigen testing has been simplified by use of an immunochromatographic card assay ([http://www.binax.com/legionella.html](#){Binax, Portland, Maine}). This assay appears to be about as sensitive and specific as the ELISA tests (Domínguez et al., 1999).

### Antimicrobial Susceptibility Testing

Because *Legionella* bacteria reside in human macrophages, there is a poor correlation between standard in vitro susceptibility tests and treatment efficacy. For example, several  $\beta$ -lactam drugs are highly active against broth grown *L. pneumophila*, but completely inactive against the intracellular bacterium, and in the treatment of Legionnaires' disease (Edelstein, 1995a). Assays of the activity of antimicrobial agents against the intracellular bacterium correlate better with clinical outcome, but may not be completely accurate. Infection of explanted guinea pig alveolar macrophages is the best-standardized model for the study of intracellular susceptibility to antimicrobial agents (Edelstein and Edelstein, 1999b). A wide variety of cell lines have been used to perform such intracellular testing, but no studies exist comparing these results to the use of the guinea pig alveolar macrophage model (Higa et al., 1998; Stout et al., 1998). Although continuous cell lines have the advantage of being readily available, their use introduces the methodologic problem of how to deal with an expanding host cell population during the course of the study and with uncertainty about antimicrobial kinetics in these cells. The best correlation between clinical effectiveness and experimental activity is with the guinea pig pneumonia model (Edelstein, 1999a).

## Environmental Ecology and Microbiology

### Environmental Ecology

The legionellae are ubiquitous in our natural aqueous environment. Most *Legionella* spp. have

been recovered only from water sources (Table 2). Humans are accidental hosts of these bacteria, which in nature appear to be facultative parasites of free-living amoebae. Early environmental surveys of natural bodies of water (including rivers, lakes and creeks) found *L. pneumophila* to be present in relatively high concentrations in most sites (Fliermans et al., 1979; Fliermans et al., 1981; Fliermans et al., 1983; Tyndall et al., 1983; Morris et al., 1979; Cordes et al., 1981; Cherry et al., 1982). In addition, thermally polluted water was more likely to contain the bacterium, especially in building hot water tanks and heaters and in water-cooled heat rejection devices such as air-conditioning cooling towers. The bacterium was found to be present in water 5–63°C (Fliermans, 1983) and to grow optimally in the environment between 25 and 40°C.

Rowbotham first proposed that *L. pneumophila* was a facultative parasite of free-living amoebae (Rowbotham, 1980a; Rowbotham, 1980b). His observations were confirmed by many other investigators (Tyndall and Domingue, 1982; Anand et al., 1983; Holden et al., 1984; Newsome et al., 1985; Henke and Seidel, 1986), and an association between outbreaks of Legionnaires' disease and the presence of amoebae was demonstrated by Fields and colleagues (Barbaree et al., 1986). The relative chlorine resistance of environmental *L. pneumophila* was explained in part by the protection afforded the bacterium growing within an amoebal cyst (Kilvington and Price, 1990), and the phenotype change of the bacterium resulting from intra-amoebal growth (Kilvington and Price, 1990; Barker et al., 1992; 1993). In addition, the growth-potentiating properties of some water specimens for *L. pneumophila* was explained by the multiplication of the bacterium in the amoebae (Yee and Wadowsky, 1982; Wadowsky et al., 1988; 1991). It is now accepted by most authorities that *L. pneumophila*, and probably most other *Legionella* spp., are facultative parasites of free-living amoebae, and that the major, if not sole form, of the organism in the environment is within amoebae. Not all *Legionella* bacteria will grow in the same amoebal host, indicating strain-to-strain variation in the selection of the optimal amoebal host (Rowbotham, 1986; Fields et al., 1989; 1990; Wadowsky et al., 1991). Of note, a number of other environmental bacteria pathogenic for man are now thought to grow within amoebae, including *Mycobacterium avium* and *Burkholderia pseudomallei* (Steinert et al., 1998; Cirillo et al., 1997; Michel and Hauröder, 1997; Marolda et al., 1999; Landers et al., 2000).

A number of biofilm studies have indicated that the bacterium grows within environmental biofilm, which contain a rich array of other bac-

teria and amoebae (Wright et al., 1989; Rogers and Keevil, 1992a; Green and Pirrie, 1993; Walker et al., 1993). The bacterium is present in highest concentration in the sessile population, but there is an equilibrium with the planktonic population (Rogers and Keevil, 1992a; Ta et al., 1995). One investigator has found that *L. pneumophila* grows in an experimental biofilm in the absence of amoebae, suggesting that the environmental growth of the organism can be quite complex (Rogers and Keevil, 1992a). Because many epidemics of Legionnaires' disease have been associated with sudden changes in water pressure within plumbing pipes, it is likely that the pressure fluxes cause release and distribution of the sessile biofilm containing large quantities of the bacterium. This is supported by one study showing a dramatic increase in *L. pneumophila* recovery after pressure shock of a plumbing system (Shands et al., 1985).

Growth of *L. pneumophila* within amoebae can enhance the virulence of the bacterium for macrophages and immunocompromised mice (Brieland et al., 1996; Cirillo et al., 1994). In addition, *L. pneumophila* grows to high concentrations within amoebae, and residence of the bacteria within the amoebae may protect the bacterium from the harmful effects of aerosolization. These facts have led to the unproven hypothesis that the infective form of the bacterium for man is via a "packet" of bacteria contained within an amoebal vacuole or cyst. A large body of literature exists regarding the molecular and cellular pathogenesis of *L. pneumophila* for amoebae, and its relationship to pathogenicity for macrophages; this topic is covered in the pathogenesis section of this review.

A viable but not cultivatable form of *L. pneumophila* has been proposed by a few investigators (Paszko-Kolva et al., 1992; Paszko-Kolva et al., 1993; Hay et al., 1995; Yamamoto et al., 1996; Steinert et al., 1997; Hussong et al., 1987). It is unclear if at least some of the reports of environmental viable but "not cultivatable" *L. pneumophila* isolates were "noncultivable" because they were in amoebae, and required prolonged incubation to amplify the small numbers present (Shahamat et al., 1991; Sanden et al., 1992). Another speculation (recently reported) is that this state represents a part of the developmental cycle of *L. pneumophila* (Garduno et al., 2000); if so the bacterium enters a vegetative cycle under different environmental conditions, which could make it easier to cultivate. Also, the significance of this phenomenon in the case of *Legionella* is uncertain. It is unknown if the viable but noncultivable bacteria, if they truly exist in nature, are pathogenic.

## Environmental Microbiologic Techniques

Somewhat different selective media and techniques must be used for isolation from environmental specimens because of differences in contaminating bacterial and fungal flora. A commonly used and useful medium is BCYE $\alpha$  medium made selective with glycine, vancomycin, polymyxin B and natamycin; this is termed "MWY" or modified Wadowsky Yee medium (Wadowsky and Yee, 1981; Edelstein, 1982a). Another useful medium is BCYE $\alpha$  medium made selective with polymyxin E, cephalothin, vancomycin and cycloheximide, termed "CCVC" medium (Bopp et al., 1981). The latter is more inhibitory than MWY medium, and is best used for heavily contaminated specimens. Addition of differential dyes is used by some, but may not be especially useful (Vickers et al., 1981). Except for rare specimens, pretreatment of the specimen before plating with either acid (KCl, pH 4, for 4 min), or heat (60°C for 1–2 min) is required (Edelstein et al., 1982d).

Optimal yield of *L. pneumophila* from tap water specimens appears to be obtained by culturing of biofilm scraped from the interior of pipes and plumbing fixtures, as opposed to free flowing water from the same devices (Ta et al., 1995). Water specimens can be concentrated before plating by either centrifugation or filtration; both methods result in the loss of substantial and unpredictable amounts of bacteria (Boulanger and Edelstein, 1995). Centrifugation at low speed (3,800  $\times$  g for 30 min) results in about 14% recovery, whereas higher speed centrifugation (8,000  $\times$  g for 15 min) results in about 30% recovery. Filtration with flat membrane filters yields about 50% recovery, whereas filtration with woven membrane filters results in about 14% recovery.

## Environmental Control and Remediation

Because *Legionella* bacteria are ubiquitous in the natural environment, as well as in many man-made environments, it is difficult (if not impossible) to eliminate the bacterium completely from many environmental sites. For the most part, this is unneeded, as the vast majority of Legionella-containing sites do not cause Legionnaires' disease. The environmental sites that are most likely to transmit the disease include wet cooling towers, evaporative condensers, whirlpool spas, hot or tepid water potable water fixtures used for bathing or washing, and any medical equipment containing tap water that can be delivered into the lungs.

Attempts at quantifying the risk of Legionnaires' disease based on the concentration of *L. pneumophila* in water are unreliable scien-

tifically, and of unproven utility ([http://www.osha-slc.gov/dts/osta/otm/otm\\_iii/otm\\_iii\\_7.html#1](http://www.osha-slc.gov/dts/osta/otm/otm_iii/otm_iii_7.html#1)) (Shelton et al., 1993; 1994). This is because of the natural variability of bacterial growth in environmental sources, the heterogeneous nature of the bacterium in biofilm, and the low sensitivity and imprecision of concentration techniques. Other factors of unknown or imprecisely known risk include the presence of susceptible hosts, adequate aerosolization of the bacterium, viability of the bacterium in aerosols, and virulence of the bacteria. It is plausible that very high concentrations of *L. pneumophila* in water sources are a higher risk than very low concentrations, but exactly what constitutes a risk is not known. Despite this, various regulatory agencies have adopted the use of "action" limits for environmental *Legionella* bacteria [www.osha-slc.gov](http://www.osha-slc.gov) ([http://www.osha-slc.gov/dts/osta/otm/otm\\_iii/otm\\_iii\\_7.html#1](http://www.osha-slc.gov/dts/osta/otm/otm_iii/otm_iii_7.html#1)); [www.env.gov.sg](http://www.env.gov.sg) (<http://www.env.gov.sg/cop/qed/remedial.html>).

Prevention of Legionnaires' disease should be based primarily on good engineering design and maintenance of plumbing systems, cooling towers, and spas. The factors important for minimizing the presence of *L. pneumophila* in plumbing systems include maintaining hot water temperatures >50°C, keeping cold water and cold water pipes <20°C, and eliminating blind loops and dead ends, thereby reducing stagnation (Brundrett, 1992; Freije, 1996; Anonymous, 1988; Chartered Institution of Building Services Engineers, 1987; Chartered Institution of Building Services Engineers, 1991; Department of Health and Social Security and the Welsh Office, 1989; Health and Safety Executive, 1991; Health and Safety Executive, 1998; Occupational Health S. A. W. C., 1995; Allegheny County Health Department, 1993; Wise et al., 1987; Brundrett, 1994). Also, elimination of hot water storage tanks, and use of water heaters with internal recirculation are important. Cooling towers require frequent cleaning, proper maintenance to control biofouling, drift eliminators, and siting of air intake ducts away from cooling tower drift (Butler et al., 1997; Anonymous, 1996; Broadbent, 1987; Brundrett, 1994). Spas require rigorous daily cleaning which includes emptying, scrubbing, and hyperchlorination, regular flushing of filters, and maintenance of an oxidizing residual at all times that is bioeffective; this requires limits on the numbers of users and hourly checks of free biocide concentration (National Center for Environmental Health and National Center for Infectious Diseases, 1995; Public Health Laboratory Service Spa Pools Working Party, 1994; Anonymous, 1985).

Whether to routinely monitor potable water and cooling tower water for the presence of *Legionella* is controversial (Yu, 1998; Breiman

and Butler, 1998; Butler et al., 1997; Tablan et al., 1994). This is because of the ubiquitous presence of the organism in most cooling tower water, and the relatively low frequency of Legionnaires' disease. Routine bacteriologic monitoring of the water distribution systems of health care facilities is least controversial, and is practiced by some health districts (Allegheny County Health Department, 1993; Anonymous, 2000).

Control of Legionella-contaminated sites can be divided into two main categories: measures to end an epidemic that implicates the particular environmental source, and measures to reduce bacterial concentration for other reasons [www.hse.gov.uk \(http://www.hse.gov.uk/pubns/misc150.pdf\)](http://www.hse.gov.uk/pubns/misc150.pdf); [www.tarn-pure.com \(http://www.tarn-pure.com\)](http://www.tarn-pure.com); [www.hcinfo.com \(http://www.hcinfo.com\)](http://www.hcinfo.com); [www.osha-slc.gov \(http://www.osha-slc.gov/dts/osta/otm/otm\\_iii/otm\\_iii\\_7.html\)](http://www.osha-slc.gov); (Brundrett, 1992; Health and Safety Commission, 1991; Health and Safety Executive, 1991; 1998; Freije, 1996). In the first category, shut down of implicated aerosol disseminators is the first step to be taken. Then application of oxidizing chemicals, usually chlorine, is instituted. In cooling towers, addition of an antifoaming agent is often needed. After the towers are disinfected, then thorough cleaning is needed. In the case of plumbing systems, extensive modifications are sometimes needed, which include elimination of dead legs, insulation of cold and hot water pipes to maintain temperatures  $<20^{\circ}\text{C}$  and  $>50^{\circ}\text{C}$ , respectively, removal and cleaning or sterilization of distal fixtures, and removal of hot water storage tanks from the plumbing systems. Once an outbreak is controlled, then continuous maintenance of effective biocide concentrations is required. In some cases, the continuous biocide used has been chlorine or chlorine dioxide. Addition of a copper-silver ionization system has been effective in many settings, but usually only when needed plumbing modifications have been made, and when an oxidizing compound is used as well (Biurrun et al., 1999; Rohr et al., 1999; Lin et al., 1998; Miuetzner et al., 1997). Hard water compromises the efficacy of the copper-silver ionization system.

## Pathogenesis

Given that legionellosis is most associated with infection by *L. pneumophila* (see above), the vast majority of studies on pathogenesis have focused on that species of *Legionella* and, in particular, strains belonging to its first serogroup. Therefore, the following description of pathogenesis should formally be viewed as an account of *L. pneumophila* (serogroup 1) pathogenesis.

The notable observations that have been made with the other *Legionella* species will be highlighted at the end of this section.

## An Overview

*Legionella pneumophila* infects humans following either the inhalation of contaminated aerosols generated by airconditioners, showers and other devices or the aspiration of contaminated potable water (Mahoney et al., 1992; Woo et al., 1992). Once delivered into the respiratory tract, the organism invades and proliferates within the resident macrophages that line the alveoli (Cianciotto et al., 1989a; Horwitz, 1992; Winn, 1988). The release of tissue-destructive substances from the bacteria undoubtedly contributes to pathology (Cianciotto et al., 1989a; Winn, 1988). As a result of this intracellular multiplication, polymorphonuclear leukocytes (PMNs), additional macrophages (i.e., differentiated monocytes), and erythrocytes infiltrate the alveoli, and capillary leakage results in local edema (Winn, 1988). This and further host defense responses are triggered, at least in part, by chemokines and proinflammatory cytokines (i.e., IL-1, IL-6, GM-CSF, MCP-3, MIP-1 $\alpha$ , MIP-2 and TNF- $\alpha$ ) released by the infected macrophages (Blanchard et al., 1987; Nakachi et al., 2000; Widen et al., 1991; Yamamoto et al., 1995). Although it is quite clear that the legionellae do not replicate within the recruited PMNs, data are still conflicting as to the ability of these phagocytes to effectively kill ingested bacteria (Blanchard et al., 1989; Fitzgeorge et al., 1988; Horwitz and Silverstein, 1981; Katz and Hashemi, 1982). In contrast, when the cell-mediated adaptive immune response is functioning normally, further bacterial amplification is usually limited (Breiman and Horwitz, 1987; Friedman et al., 1998; Horwitz, 1992; Klein et al., 1991; Skerrett and Martin, 1992). Most evidence points to a critical role for the Th1 T-cell response and its associated cytokines (e.g.,  $\gamma$ -interferon [ $\gamma$ -IFN] and interleukin-12) in the clearance of *L. pneumophila* (Brieland et al., 1998; Heath et al., 1996; Kitsukawa et al., 1995; Newton et al., 2000; Skerrett and Martin, 1994; Susa et al., 1998; Tateda et al., 1998). Indeed,  $\gamma$ -IFN activation of macrophages renders the cells nonpermissive for the *Legionella* parasite (Bhardwaj et al., 1986; Fujio et al., 1992; Jensen et al., 1987; Klein et al., 1991; Nash et al., 1988; Skerrett and Martin, 1992; Watanabe et al., 1993). When adaptive immune response is impaired in certain immunocompromised individuals, bacterial proliferation and extrapulmonary dissemination are pronounced, and disease may be fatal (Doebbeling and Wenzel, 1987; Skerrett and Martin, 1992; Winn, 1988).

Animal models have been an invaluable tool for determining the basic course of an *L. pneumophila* infection, the role of particular bacterial factors in pathogenesis, and the basis of host defense (Cianciotto et al., 1989a; Collins, 1986). Legionellosis is most often reproduced by infection of guinea pigs, corticosteroid-treated rats, or A/J mice, using the aerosol or intratracheal routes of inoculation (Baskerville et al., 1983; Berendt et al., 1980; Brieland et al., 1994; Davis et al., 1982; 1983; Edelstein et al., 1984; Skerrett et al., 1989; Susa et al., 1998; Twisk-Meijssen et al., 1987; Winn et al., 1982). Interestingly, a number of inbred mice are not susceptible to *L. pneumophila*, and thus crosses between them and the A/J animals are being used to identify mammalian loci (e.g., *Lgn1*) that influence host susceptibility (Beckers et al., 1995; Dietrich et al., 1995; Diez et al., 1997; Growney et al., 2000; Yamamoto et al., 1992; Yoshida et al., 1991).

### The Relative Importance of Intracellular Infection and Extracellular Survival

Much evidence indicates that the capacity of the *L. pneumophila* to grow within alveolar macrophages is central to the pathogenesis of Legionnaires' disease. First, the majority of bacteria observed in lung samples from infected humans and animals are associated with these cells (Chandler et al., 1979a; Davis et al., 1983; Glavin et al., 1979; Katz et al., 1979; Rodgers et al., 1978; Surgot et al., 1988; Watson and Sun, 1981; White et al., 1979). Second, *L. pneumophila* readily replicates within alveolar macrophages and monocytes in vitro (Horwitz and Silverstein, 1980; Jacobs et al., 1984; Kishimoto et al., 1979; Nash et al., 1984). Third, the susceptibility of an animal species to infection is correlated with the ability of *L. pneumophila* to grow within macrophages from that species (Yamamoto et al., 1987; Yamamoto et al., 1988; Yoshida and Mizuguchi, 1986). Fourth, the resistance of animals to infection requires  $\gamma$ -IFN, the cytokine that activates macrophages (Brieland et al., 1994; Skerrett and Martin, 1994). Fifth, mutants that are impaired in their ability to infect macrophages exhibit reduced virulence (Cianciotto et al., 1990b; Edelstein et al., 1999c; Jacobs et al., 1984; Liles et al., 1999; Marra et al., 1992; Pearlman et al., 1988; Viswanathan et al., 2000). Finally, as noted earlier, therapy requires antibiotics that enter eukaryotic cells (Winn, 1988).

Despite the early recognition of the Legionella-macrophage interaction, it was not immediately obvious how *L. pneumophila*, an organism that neither possesses a mammalian reservoir nor exhibits a "natural" route of infection, evolved the facility to parasitize human

phagocytes. It is now believed that adaptation to intracellular niches within protozoa engendered in *L. pneumophila* the ability to infect mammalian cells (Atlas, 1999; Cianciotto and Fields, 1992; Fields, 1996; Harb et al., 2000b; Swanson and Hammer, 2000). Several observations signal that the requirements for growth within macrophages mimic those in protozoa. First, the intracellular pathway pursued by the *Legionella* parasite, which includes replication within a membrane-bound vesicle (phagosome), is remarkably similar in protozoa and macrophages (Abu Kwaik, 1996a; Bozue and Johnson, 1996; Horwitz, 1983a; Newsome et al., 1985; Rowbotham, 1986). Second, bacteria grown in amoebae maintain their ability to infect macrophages and to cause disease (Steinert et al., 1994; Vandenesch et al., 1990). Indeed, amoeba-grown *L. pneumophila* are more infective for macrophages and epithelial cells than are agar-grown organisms (Cirillo et al., 1994). Third, strains that have been rendered avirulent by passage on artificial media are unable to infect both protozoa and macrophages (Fields et al., 1986; Rowbotham, 1986). Finally, as a form of genetic proof, a variety of mutants are similarly impaired in their ability to infect macrophages and protozoans (Cianciotto and Fields, 1992; Gao et al., 1997; Liles et al., 1999; Segal and Shuman, 1999a). Hence, a number of virulence factors likely evolved in response to selective pressures within the protozoan environment.

Without diminishing the significance of growth within macrophages, it is likely that other factors also contribute to the survival of *L. pneumophila* within humans. For example, the bacterium may replicate or, at a minimum, must survive within extracellular spaces in the alveoli (Chandler et al., 1979a; Rodgers et al., 1978; Surgot et al., 1988; Watson and Sun, 1981; White et al., 1979). The fact that many strains of *L. pneumophila* are inherently serum resistant may be particularly relevant for extracellular survival following the onset of the inflammatory response (Caparon and Johnson, 1988; Horwitz and Silverstein, 1981; Luneberg et al., 1998; Plouffe et al., 1985; Verbrugh et al., 1985). Similarly, the ability of the bacterium to resist cationic peptides and to secrete enzymes that degrade surfactant suggests that it might subvert antimicrobial factors released by lung epithelia (M. Robey, W. O'Connell and N. P. Cianciotto, 2001; Edelstein, 1981; Flieger et al., 2000b; Wadowsky and Yee, 1981). The presence of legionellae within non-macrophage cells in necropsy material suggests that *L. pneumophila* also may grow within the alveolar epithelium (Rodgers, 1979; Watson and Sun, 1981). In support of this notion, the microbe can replicate within alveolar types I and II cells, as well as a variety of other epithelial cells in



vitro (Cianciotto et al., 1995; Mody et al., 1993). The importance of extra-macrophage niches or processes is also implied by two additional observations. First, those *L. pneumophila* clone types that most often represent the clinical isolates (which are MAb-2 positive) are not necessarily more effective at intracellular infection (Edelstein and Edelstein, 1993b). Second, some genetically defined mutants that are not defective or only moderately defective for macrophage infection in vitro are strikingly impaired in animal models of virulence (Edelstein et al., 1999c; Liles et al., 1999).

### The Intracellular Infection Process

Given the pivotal role that intracellular parasitism plays in the biology of *L. pneumophila*, a first-line approach toward understanding legionellosis has been to study the cellular basis of macrophage infection (Abu Kwaik, 1998a; Cianciotto et al., 1989a; Horwitz, 1992; Ott, 1994; Roy, 1999; Shuman et al., 1998; Swanson and Hammer, 2000; Vogel and Isberg, 1999). This effort has been aided enormously by the availability of human macrophage-like cell lines, such as U937, HL-60, Mono Mac, and THP-1 cells (Cirillo et al., 1994; Marra et al., 1990; Neumeister et al., 1997; Pearlman et al., 1988).

Initially, strains of *L. pneumophila* enter the macrophage by conventional or coiling phagocytosis, processes that are dependent on the host cell actin cytoskeleton (Elliott and Winn, 1986; Horwitz, 1984a; King et al., 1991; Rechnitzer and Blom, 1989). Some studies indicate that phagocytosis involves opsonization of the bacteria with the complement component C3 and the subsequent binding to complement receptors CR1 and CR3 (Payne and Horwitz, 1987). Entry via this pathway appears to limit the phagocyte's oxidative burst and thereby may help to facilitate subsequent bacterial intracellular survival (Jacob et al., 1994). Other reports, however, have stressed the importance of opsonin-independent phagocytosis (Gibson et al., 1994; Husmann and Johnson, 1992; Rodgers and Gibson, 1993; Steinert et al., 1994). When the legionellae are grown in amoebae, as opposed to bacteriologic media, their entrance into monocytes is more likely to involve complement-independent, coiling phagocytic events (Cirillo et al., 1999). Although this alternate mode of entry may elicit an oxidative burst, it should be noted that a number of *L. pneumophila* strains exhibit innate resistance to hydrogen peroxide, superoxide anion, and hydroxyl radicals (Jepras and Fitzgeorge, 1986; Lochner et al., 1983). After entry into the macrophage host is complete, the legionellae reside within a phagosome that is unusual for its hypoexpression of major histocompatibility

(MHC) antigens (Clemens and Horwitz, 1992; Clemens and Horwitz, 1993). This nascent phagosome does not fuse with early or late endosomes or lysosomes (Clemens and Horwitz, 1995; Clemens et al., 2000; Horwitz, 1983b). As a result of this form of phagosome trafficking, *L. pneumophila* escapes the killing that is promoted by vacuolar acidification and lysosomal degradation and reduces or delays the association of its protein with MHC Class II that is required for antigen presentation (Clemens and Horwitz, 1995; Horwitz, 1983b; Horwitz and Maxfield, 1984b; Wiater et al., 1998). Within 4–12 h post-infection, mitochondria, smooth vesicles and rough endoplasmic reticulum surround the *L. pneumophila* phagosome, a process that is orchestrated by the bacterial parasite (Coers et al., 1999; Glavin et al., 1979; Horwitz, 1983a; Swanson and Isberg, 1995). A recent study suggests that, late in the intracellular cycle (i.e., 12–24 h), the *Legionella* phagosome does fuse with acidic lysosomal compartments, but bacterial growth continues (Sturgill-Koszycki and Swanson, 2000). Within its specialized niches, the legionellae replicate by binary fission, creating a large phagosome that fills much of the host cell (Abu Kwaik, 1996a; Horwitz, 1983a). Intracellular replication itself is significantly influenced by host cell iron and thymidine levels (Byrd and Horwitz, 1989; Byrd and Horwitz, 1991; Byrd and Horwitz, 2000; Gebran et al., 1994; Hickey and Cianciotto, 1997; Mengaud and Horwitz, 1993; Mintz et al., 1988; Pope et al., 1996; Stone et al., 1999b). Ultimately, *L. pneumophila* intracellular infection results in the death and lysis of the host cell. Macrophage death appears to involve an early induction of caspase-3 mediated apoptosis and a late form of necrosis triggered by a bacterial pore-forming activity (Alli et al., 2000; Gao and Abu Kwaik, 1999a; Gao and Abu Kwaik, 1999b; Hagele et al., 1998; Muller et al., 1996). In contrast to the series of events just outlined, exposure of a macrophage to large numbers of attached (extracellular) *L. pneumophila* results in immediate pore formation in the host cell plasma membrane and rapid necrotic cell death (Husmann and Johnson, 1994; Kirby et al., 1998; Zuckman et al., 1999).

### Virulence Factors of *L. pneumophila*

Microbiological, biochemical, ultrastructural and genetic approaches have identified some of the factors that may potentiate *L. pneumophila* intracellular infection and/or virulence (Abu Kwaik, 1998a; Belyi, 1999; Cianciotto et al., 1989a; Dowling et al., 1992; Hacker et al., 1993; Swanson and Hammer, 2000). Among the genetic tools for manipulating *L. pneumophila* are the methods of conjugal transfer from

recombinant *Escherichia coli*, electroporation of recombinant plasmids, allelic exchange (directed) mutagenesis, random mutagenesis with mini-Tn10 transposons, shuttle and signature-tagged mutagenesis, reporter gene fusions, and differential display (Abu Kwaik and Pederson, 1996b; Albano et al., 1992; Chen et al., 1984; Cianciotto and Fields, 1992; Cianciotto et al., 1988; Dreyfus and Iglewski, 1985; Edelstein et al., 1999c; Engleberg et al., 1988; Hickey and Cianciotto, 1997; Kohler et al., 2000; Marra and Shuman, 1989; McClain and Engleberg, 1996a; Mintz and Shuman, 1987; Pope et al., 1994; Tully et al., 1992; Wiater et al., 1994a; 1994b).

One of the earliest observations concerning *L. pneumophila* virulence was the finding that legionellae passaged on bacteriologic media had the potential to be converted to an avirulent state (Elliott and Johnson, 1982; McDade and Shepard, 1979). This virulent to avirulent conversion is heavily influenced by the nature of the medium; e. g., high-salt supplemented-Mueller-Hinton agar favors the growth of avirulent forms, whereas growth of the virulent strain can be maintained on buffered charcoal yeast extract (BCYE) agar so long as a reasonable passage number is not exceeded (Catrenich and Johnson, 1988; Catrenich and Johnson, 1989; Horwitz, 1987; Nowicki et al., 1987; Yamamoto et al., 1993). The second factor to potentiate the ability of *L. pneumophila* to infect human cells is its growth phase; i. e., stationary phase bacteria are more infective than are exponential phase organisms (Byrne and Swanson, 1998). This conversion in phenotype is triggered by amino acid depletion and involves an intracellular accumulation of ppGpp, the classic indicator of the bacterial stringent response (Hammer and Swanson, 1999). Iron limitation also reversibly diminishes the virulence of *L. pneumophila*, signaling the importance of iron uptake in *Legionella* pathogenesis (see below; James et al., 1995). Finally, growth temperature has been found to have a variable and relatively minor influence on *L. pneumophila* virulence and intracellular infectivity (Berg et al., 1985; Edelstein et al., 1987b; Mauchline et al., 1994).

As is often the case for bacterial pathogens, a variety of *L. pneumophila* surface structures and molecules have been implicated in pathogenesis. The *L. pneumophila* LPS, in addition to bearing the serogroup-specific O antigen (see above), contains endotoxin (Ciesielski et al., 1986; Conlan and Ashworth, 1986a; Highsmith et al., 1978; Nolte et al., 1986; Otten et al., 1986). However, the LPS-lipid A of *Legionella* possesses a relatively weak endotoxic activity that appears to be due to its low affinity for the CD14 receptor on macrophages (Neumeister et al., 1998a; Schramek et al., 1982; Wong et al., 1979). Inter-

estingly, a particular LPS epitope, which is recognized by the typing MAb 2, is more frequently expressed on clinical versus environmental isolates of serogroup 1 strains (Dournon et al., 1988; Helbig et al., 1995; Zou et al., 1999). However, mutational analysis has determined that loss of that epitope itself does not diminish the ability of *L. pneumophila* to infect macrophages or protozoa (Mintz and Zou, 1992b). On the other hand, several recent studies have correlated other antigenic changes in LPS expression with reductions in serum resistance, intracellular growth, and virulence (Luneberg et al., 1998; Rogers et al., 1992b). Finally, mutation of a gene (*rcp*) that appears to modify lipid A structure reduces the ability of *L. pneumophila* to resist cationic antimicrobial peptides (i. e., polymyxin and defensin C18G) and to infect host cells (M. Robey, W. O'Connell, and N. P. Cianciotto, manuscript submitted). A second prominent surface feature of *L. pneumophila* is its flagella (Chandler et al., 1980; Rodgers et al., 1980a). The gene encoding the flagellin subunit has been cloned, sequenced, and found to be regulated by temperature, growth phase, amino acids, viscosity and osmolarity (Hammer and Swanson, 1999; Heuner et al., 1995; Heuner et al., 1999; Ott et al., 1991). Although mutational analyses indicate that *Legionella* flagella are not required for intracellular replication per se, they appear to promote bacterial entry into host cells (Merriam et al., 1997; Pruckler et al., 1995a; Dietrich et al., 2000; Ross et al., 2000). *Legionella pneumophila* have at least two types of pili (Chandler et al., 1980; Rodgers et al., 1980a; Stone and Abu Kwaik, 1998). One form includes the temperature-regulated, bundle-forming type IV pili, which facilitate to a modest degree bacterial attachment to host cells (Liles et al., 1998; Stone and Abu Kwaik, 1998). A fourth *L. pneumophila* surface structure is the genus-wide, peptidoglycan-linked, outer membrane porin (Gabay et al., 1985; Hoffman et al., 1992a; Hoffman et al., 1992b). Notably, this 28-kDa protein, which is also known as the major outer membrane protein (MOMP) of *L. pneumophila*, is a binding site for complement components and thus mediates opsonophagocytosis (Bellinger-Kawahara and Horwitz, 1990). The 24-kDa Mip is another genus-wide, surface-exposed membrane protein, which exists as a homodimer and possesses prolyl-proline isomerase (PPIase) activity (Cianciotto et al., 1990a; Engleberg et al., 1989; Fischer et al., 1992; Riffard et al., 1996; Schmidt et al., 1994). The *mip* gene is required for the early stages of intracellular infection of macrophages, protozoa, and lung epithelia and for virulence following intratracheal inoculation of guinea pigs (Cianciotto et al., 1989b; 1990b; 1995; Cianciotto and Fields, 1992; Susa et al., 1996; Winter-

meyer et al., 1995). The analysis of Mip, in addition to providing for the first definition of a *Legionella* virulence factor, has now yielded the first crystal structure of a *Legionella* protein (Cianciotto et al., 1990b; Hilgenfeld et al., 2000). The final surface proteins that have received some attention are a 19-kDa outer membrane lipoprotein, a 25-kDa MOMP and a 60-kDa heat shock protein (Hsp60; Garduno et al., 1998a; High et al., 1993; Hindahl and Iglewski, 1987; Hoffman et al., 1990; Sampson et al., 1990). Although the genus-wide, lipoprotein gene has been cloned and sequenced (Engleberg et al., 1991; Ludwig et al., 1991), examination of its role in pathogenesis has not been forthcoming. On the other hand, data suggest that the 25-kDa protein promotes bacterial attachment to a macrophage-like cell line (Krinis et al., 1999). The Hsp60 is induced upon intracellular infection and appears to promote epithelial cell invasion (Abu Kwaiq et al., 1993; Fernandez et al., 1996; Garduno et al., 1998b). Furthermore, it elicits proinflammatory cytokine (e.g., IL-1) expression by macrophages (Retzlaff et al., 1994; 1996).

Much work has highlighted the importance of *Legionella* protein secretion systems for pathogenesis. For some time, it has been known that *L. pneumophila* secretes a variety of degradative enzymes, when it is grown in or on bacteriologic media (Baine et al., 1979b; Baine, 1985; Müller, 1981; Nolte et al., 1982; Thorpe and Miller, 1981). Recent studies indicate that many of these factors, including two acid phosphatases, an RNase, a zinc metalloprotease, a monoacylglycerol lipase, a phospholipase A, a lysophospholipase A, and a *p*-nitrophenylphosphorylcholine hydrolase (previously, viewed as a phospholipase C), are secreted via a type II protein secretion system, which is itself dependent upon the type IV prepilin peptidase (PilD; O. Rossier and N. P. Cianciotto, 2001; Aragon et al., 2000; Aragon et al., 2001; Flieger et al., 2000a; Hales and Shuman, 1999a; Liles et al., 1998; Liles et al., 1999). Mutations within the genes encoding the secretion system reduce the ability of *L. pneumophila* to infect both human macrophages and protozoa, suggesting that one or more of the type II exoproteins are virulence determinants (O. Rossier and N. P. Cianciotto, manuscript submitted; Hales and Shuman, 1999a). The behavior of mutants specifically defective in single exoproteins has thus far eliminated essential intracellular infection roles for the metalloprotease, the major acid phosphatase, and the *p*-nitrophenylphosphorylcholine hydrolase (Aragon et al., 2000; Aragon et al., 2001; Moffat et al., 1994; Szeto and Shuman, 1990). The metalloprotease is at best a weak virulence factor in vivo, inhibiting the chemotactic response and oxidative burst of neutrophils, promoting some

pulmonary damage and cell cytotoxicity, and degrading a variety of substrates such as IL-2 (Baskerville et al., 1986; Blander et al., 1990; Conlan et al., 1988a; Dreyfus and Iglewski, 1986; Edelstein et al., 1999c; Mintz et al., 1993; Moffat et al., 1994; Müller, 1980; Quinn and Tompkins, 1989; Rechnitzer and Kharazmi, 1992; Sahney et al., 1990; Williams et al., 1987). *Legionella pneumophila* does possess a variety of proteases, and presumably some of them can, if necessary, function in the absence of the zinc metalloprotease (Berdal and Olsvik, 1983; Conlan et al., 1986b; Conlan et al., 1988b; Gul'nik et al., 1986). Interestingly, a mutation in the PilD peptidase gene leads to both a sharp reduction in animal virulence and a more dramatic reduction in intracellular infection than do mutations of genes for either the type II secretion system or the type IV pilus (O. Rossier and N. P. Cianciotto, manuscript submitted; Liles et al., 1999). These data suggest the existence of another, potentially novel secretion pathway that promotes bacterial pathogenesis. A second type of protein secretion system that clearly exists within *L. pneumophila* and promotes intracellular infection is a type IV system (Christie and Vogel, 2000). This system, which is dependent upon a large number of genes contained within the so-called "defective for organelle trafficking" (*dot*) and "intracellular multiplication" (*icm*) loci, is critical for the ability of *L. pneumophila* to inhibit phagosome-endolysosomal fusions and to establish its unique replicative niche (Andrews et al., 1998; Berger and Isberg, 1993; Berger et al., 1994; Brand et al., 1994; Marra et al., 1992; Purcell and Shuman, 1998; Sadosky et al., 1993; Segal et al., 1998b; Segal and Shuman, 1997; Vogel et al., 1998). Several *icm/icm* loci also have been shown to be essential for virulence in a guinea pig model of disease (Edelstein et al., 1999c; Marra et al., 1992). Interestingly, the Dot/Icm secretion system is also structurally and functionally homologous to bacterial conjugation systems (Segal and Shuman, 1998a; Segal et al., 1998b; Vogel et al., 1998). Among the factors believed to constitute the secretion apparatus, DotA is clearly an inner membrane protein that plays a critical role in the initial trafficking of the *Legionella* phagosome (Roy et al., 1998; Roy and Isberg, 1997; Swanson and Isberg, 1996; Wiater et al., 1998). Aside from the IcmX protein, the factors secreted by the *Legionella* Dot/Icm system remain largely unknown (Matthews and Roy, 2000; Zuckman et al., 1999). Recently, a second *L. pneumophila* type IV secretion system (i.e., the *lvh* system) was identified, although it proved not to be critical for intracellular infection (Segal et al., 1999b). One of the earliest described, putative virulence factors of *L. pneumophila* is a heat-stable peptide toxin that appears in culture

supernatants (Friedman et al., 1980; Hedlund, 1981). Although partially purified peptide toxin was found to inhibit the oxidative burst of PMNs (Friedman et al., 1982; Lochner et al., 1985), the inability to purify the toxin and/or define its genetic basis have prevented an assessment of its overall importance for pathogenesis (Dowling et al., 1992). The most obvious secreted product of *L. pneumophila* is actually not a protein but rather a brown pigment, a polymer of homogentisic acid whose production requires a tyrosine-containing medium and the genus-wide legiolyisin (*lly*) gene (Baine and Rasheed, 1979a; Bender et al., 1991; Wintermeyer et al., 1991). Mutants in *lly*, however, are not impaired in intracellular infection, although they are, interestingly, hypersensitive to light (Steinert et al., 1995; Wiater et al., 1994b; Wintermeyer et al., 1994).

Several putative virulence factors, especially stress-response enzymes, have been localized to the *L. pneumophila* periplasm or cytoplasm or both (Pine et al., 1984). A copper-zinc superoxide dismutase (SOD) resides in the *Legionella periplasm*, affording resistance to toxic superoxide anions (by converting them to H<sub>2</sub>O<sub>2</sub>, see below) and promoting survival during stationary phase (St. John and Steinman, 1996). Mutational analysis indicates, however, that the enzyme is not required for bacterial infection of macrophages. A second SOD, which bears iron as its cofactor and exists within the *Legionella* cytoplasm, is essential for bacterial viability, and thus its role in pathogenesis cannot be assessed by the genetic approach (Sadosky et al., 1994; Steinman, 1992).

*Legionella pneumophila* possesses two catalase-peroxidases, enzymes that convert H<sub>2</sub>O<sub>2</sub> to innocuous water and oxygen (Bandyopadhyay and Steinman, 1998). The KatA catalase-peroxidase, like the CuZn-SOD, is located in the periplasm and is induced during stationary phase (Amemura-Maekawa et al., 1999). The KatB enzyme is cytoplasmic, and, importantly, recent mutational analysis has identified a role for the *katB* gene in intracellular infection (Bandyopadhyay and Steinman, 1998). A third type of enzyme that is associated with the *L. pneumophila* periplasm is an alkaline phosphatase (Kim et al., 1994). However, mutants that are defective in the production of the enzyme are not impaired in intracellular infection of macrophages. The *Legionella hel* locus, which is predicted to encode an inner membrane heavy metal transporter, also is not required for intracellular growth, but does appear to promote host cytotoxicity (Arroyo et al., 1994; McClain et al., 1996b). In addition to the surface-associated Mip (see above), an *L. pneumophila* PPIase, known as Cyp18, has been identified in the bacterial cyto-

plasm (Schmidt et al., 1996). Because the mutant strain defective for Cyp18 has been only tested in amoebal infection, the importance of this second PPIase for pathogenesis remains unclear. The *L. pneumophila* global stress protein GspA, stationary-phase  $\sigma$  factor RpoS, pyrophosphatase Ppa, and the novel LigA protein are induced by stress and macrophage infection (Abu Kwaik, 1998b; Abu Kwaik and Engleberg, 1994; Hales and Shuman, 1999b; Susa et al., 1996). Mutational analysis has determined that at least GspA, RpoS and LigA are not required for macrophage infection (Abu Kwaik et al., 1997; Fettes et al., 2000). Finally, the *L. pneumophila* *oad* (oxaloacetate decarboxylase), *asd* (aspartate- $\beta$ -semialdehyde), *pts* (phosphoenolpyruvate phosphotransferase) and *prp* (propionate catabolism) genes enhance intracellular infection (Edelstein et al., 1999c; Harb and Abu Kwaik, 1998; Jain et al., 1996; Stone et al., 1999b; Higa and Edelstein, 2000).

Recent screens of randomly mutagenized legionellae as well as differential display-PCR have uncovered a large number of other genes that promote intracellular infection (Abu Kwaik and Pederson, 1996b; Cirillo et al., 2000; Gao et al., 1997; Gao et al., 1998; Pope et al., 1996; Swanson and Isberg, 1996). Collectively, these loci promote all the various stages of intracellular infection; i.e., entry, intracellular survival and trafficking, replication, and escape. Whereas some of these genes (e.g., the phosphomannose isomerase [*pmi*] gene) promote protozoan and macrophage infection to similar degrees, others (e.g., macrophage-specific infectivity locus [*mil*]) are particularly important for the macrophage infection (Gao et al., 1997; Gao et al., 1998). For the most part, the structure, location and function of the corresponding gene products are unknown. However, the early macrophage locus (*eml*), enhanced entry (*enh*), and *milA* genes appear to encode novel proteins that are important during the early stages of infection, including entry and the formation of the replicative phagosome (Abu Kwaik and Pederson, 1996b; Cirillo et al., 2000; Harb and Abu Kwaik, 2000a). Other genes that are now known as the *rib* loci may encode a pore-forming toxin, which is associated with bacterial escape from the dying macrophage (Alli et al., 2000).

As can be appreciated from the above discussion of virulence factors, most research efforts have targeted those determinants involved in bacterial attachment, evasion of host defense, and tissue damage. Thus, aspects of bacterial nutrient acquisition and replication (*per se*) in vivo and in host cells remain largely unknown, as is often the case in analyses of other pathogens. However, one form of nutrient acquisition that has been addressed and is necessary for

pathogenesis is iron assimilation (V. K. Viswanathan and N. P. Cianciotto, 2001; Muller et al., 1983; Viswanathan et al., 2000; Owens et al., 1982). As expected, *L. pneumophila* iron acquisition is both important for intra- and extracellular replication and involves secreted and cell-associated factors. First, the organism excretes a siderophore, a high-affinity, nonproteinaceous ferric iron chelator that is unique in structure and has been designated as "legiobactin" (Liles et al., 2000). Because the genes encoding legiobactin are presently unknown, the role of the siderophore in intracellular infection and virulence has been difficult to gauge. On the other hand, genetic evidence does indicate that *L. pneumophila* produces a second siderophore, which is a hydroxymate-like molecule that may promote infection of macrophages (Hickey and Cianciotto, 1997). Analysis of another class of mutants indicates that *L. pneumophila* ferric iron acquisition and intracellular infection are also facilitated by genes encoding a unique methyltransferase (*iraA*), a membrane (iron-)peptide transporter (*iraB*), and the inner-membrane cytochrome *c* biogenesis system (*ccm*; V. K. Viswanathan et al., manuscript submitted; Pope et al., 1996; Viswanathan et al., 2000). Periplasmic and cytoplasmic ferric reductases aid in further iron assimilation, although their role in pathogenic processes has not been investigated (James et al., 1997; Johnson et al., 1991; Poch and Johnson, 1993). Given that bacterial pathogens usually access multiple iron sources, it is notable that *L. pneumophila* also can utilize heme-containing compounds (O'Connell et al., 1996). However, mutation of a *L. pneumophila* gene (*hbp*) that promotes heme binding does not diminish intracellular infection (O'Connell et al., 1996). Some of the *L. pneumophila* iron acquisition genes appear to be controlled by ferric uptake regulator (Fur), a transcriptional regulator that senses intracellular iron levels (Hickey and Cianciotto, 1994; 1997; O'Connell et al., 1996). *Legionella* strains do not directly utilize lactoferrin or transferrin, two host iron chelators that are used by a number of other pathogens. In the case of lactoferrin, the legionellae can bind the host factor, but the interaction is bactericidal (Bortner et al., 1986; 1989). In the case of transferrin, the bacteria are simply incapable of binding the iron chelator (Goldoni et al., 1991; James et al., 1997; Johnson et al., 1991). It should be noted, however, that transferrin may be an indirect iron source for *L. pneumophila*; i.e., *Legionella* protease(s) can degrade transferrin, resulting in the release of ferric iron (James et al., 1997).

It is perhaps not surprising that the virulence determinants thus far identified or hypothesized are all involved, at least partially, in intracellular

infection. However, a recent study, which employed signature-tagged mutagenesis, has uncovered genes that appear relevant for non-macrophage related virulence (Edelstein et al., 1999c). Further analysis of these so-called *nmv* genes should prove quite interesting (Edelstein et al., 2000).

### Pathogenic Aspects of *Legionella* Species Other Than *L. pneumophila*

Approximately one half of the *Legionella* species have been associated with human disease (see above). Many of these species, as well as some of the species that as yet have not been linked to cases of legionellosis, have the ability to infect and grow within macrophages (Fields et al., 1990; Arata et al., 1992; Izu et al., 1999; Levi et al., 1987; Maruta et al., 1998; Miyamoto et al., 1996; Neumeister et al., 1997; Weinbaum et al., 1984). Among the non-pneumophila species, *L. micdadei* has been examined the most for cellular and molecular aspects of intracellular infection (Dowling et al., 1992; Saha et al., 1988; Saha et al., 1989). Although strains of *L. micdadei* possess a functional Mip protein and induce apoptosis in and cytokine release by host cells (Gao et al., 1999c; Neumeister et al., 1998b; O'Connell et al., 1995), their mode of macrophage infection is notably different from that of *L. pneumophila*. For example, they, for the most part, do not reside within phagosomes that evade lysosomes and recruit rough endoplasmic reticulum (Gao et al., 1999c; Joshi and Swanson, 1999; Rechinzer and Blom, 1989; Weinbaum et al., 1984). Interestingly, *L. dumoffii* partly multiplies free within the cytoplasm of non-macrophage hosts (Maruta et al., 1998). Thus, as the various legionellae become more associated with human disease, it will become increasingly important for researchers to examine them carefully and not assume that they are simple equivalents of *L. pneumophila*. Indeed, the emergence of *L. longbeachae* as a serious health threat in Australia has prompted initial inquiries into that species' infectivity determinants (Doyle et al., 1998).

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# The Genus *Haemophilus*

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## Introduction

All members of the genus *Haemophilus* are small, pleomorphic, Gram-negative coccobacilli. The genus consists of diverse species, including some that are pathogens in humans and others that are pathogens in animals. Additional species are commensal organisms and are not associated with disease. The type species is *H. influenzae*, which was first isolated during the 1889 influenza pandemic and was originally believed to be the causative agent of influenza.

## Taxonomy

Over the years, the taxonomy of the genus *Haemophilus* has been subject to considerable revision. At one time *Haemophilus* was included in the family Brucellaceae. In the eighth edition of {*Bergey's Manual of Determinative Bacteriology*} (Zinnemann and Biberstein, 1974), this genus was grouped with "Genera of Uncertain Affiliation." Since 1984, *Haemophilus* has been included along with *Actinobacillus* and *Pasteurella* in the family Pasteurellaceae (Kilian and Biberstein, 1984).

Traditionally *Haemophilus* species were defined based on the requirement for factor X or factor V or both for aerobic growth. However, in the present classification scheme, some species can grow without either of these factors. Furthermore, dependence on factor X or factor V is not definitive for *Haemophilus*, as some species of *Actinobacillus* and *Pasteurella* also require factor V.

As summarized in Table 1, *H. influenzae* (including biogroup aegyptius) and *H. haemolyticus* require both factor X and factor V, *H. parainfluenzae*, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. paraphrophilus*, *H. segnis*, *H. parasuis* and *H. paracuniculus* require factor V only, and *H. ducreyi* and *H. haemoglobinophilus* require factor X only. *Haemophilus paragallinarum* usually requires factor V, but factor V-independent *H. paragallinarum* strains also have been described and account for the

vast majority of isolates in certain geographic regions (Mifflin et al., 1995). Similarly, *H. aphrophilus* sometimes requires factor X for primary isolation, but thereafter usually grows independently of both factor X and factor V. *Haemophilus somnus* and *H. agni* are closely related organisms that resemble the species *Histophilus ovis* and do not require either factor X or factor V; these organisms have not been validated as *Haemophilus* species and are referred to as species incertae sedis (of uncertain or doubtful affiliation). Some authors have suggested that *H. somnus* and *H. agni* be transferred to the genus *Histophilus*. Organisms that were formerly included in the genus *Haemophilus* include *Actinobacillus pleuropneumoniae* and *Taylorella equigenitalis*.

Beside the requirement for factor X or factor V or both, other criteria that facilitate distinction of one *Haemophilus* species from another include the ability to lyse horse erythrocytes, the presence of catalase, and the pattern of sugar fermentation (Table 1). Strains of *H. influenzae* and *H. parainfluenzae* can be subdivided into biotypes based on biochemical reactions that determine the presence of urease and ornithine decarboxylase, and the production of indole (Table 2). Strains of *H. influenzae* can be further classified according to their polysaccharide capsule. There are a total of six structurally and antigenically distinct capsular types (referred to as serotypes), designated a through f (Table 3). In addition, strains can be nonencapsulated, as defined by their failure to react with typing antisera to all capsular serotypes, and are considered nontypeable. Most type b isolates are biotype I, whereas the majority of nontypeable strains are biotype II or III.

In recent years, multilocus enzyme electrophoresis has been employed to examine isolates of *H. influenzae*. Studies with this technique have demonstrated that the population structure of encapsulated *H. influenzae* is clonal, with most isolates falling into a few common clonal groups (Musser et al., 1990; Musser et al., 1988). Nontypeable strains are genetically distinct and are more heterogeneous compared

Table 1. Differential characteristics of *Haemophilus* species.

Organism	X factor required	V factor required	Hemolysis of horse blood	Catalase	Fermentation of							
					Glucose	Fructose	Sucrose	Lactose	Xylose	Ribose	Mannose	
<i>H. influenzae</i>	+	+	-	+	+	-	-	-	-	+	+	-
<i>H. parainfluenzae</i>	-	+	-	d	+	+	+	-	-	-	-	+
<i>H. haemolyticus</i>	+	+	+	+	+	w	-	-	d	+	+	-
<i>H. parahaemolyticus</i>	-	+	+	d	+	+	+	-	-	-	-	-
<i>H. paraphrohaemolyticus</i>	-	+	+	+	+	+	+	+	-	-	-	-
<i>H. aphrophilus</i>	-	-	-	-	+	+	+	+	+	-	+	+
<i>H. paraphrophilus</i>	-	+	-	-	+	+	+	+	+	-	+	+
<i>H. segnis</i>	-	+	-	d	w	w	-	w	-	-	-	-
<i>H. ducreyi</i>	+	-	-	-	[d]	-	-	-	-	-	-	-
<i>H. paracuniculus</i>	-	+	-	+	+	+	-	+	-	-	nd	nd
<i>H. paragallinarum</i>	-	+	-	-	+	+	-	+	d	+	+	+
<i>H. parasuis</i>	-	+	-	+	+	+	-	+	-	+	+	+
<i>H. haemoglobinophilus</i>	+	-	-	+	+	-	-	+	d	-	d	+
" <i>H. somnus</i> "	-	-	-	-	+	+	-	-	-	+	+	+
" <i>H. agni</i> "	-	-	-	-	+	+	-	-	-	+	nd	+
					+	+	-	-	-	w	nd	-

Symbols: +, yes; -, no; d, differences encountered; [d], delayed positive reaction; w, weakly positive reaction; nd, not determined.

Table 2. Distinguishing characteristics of *H. influenzae* and *H. parainfluenzae* biotypes.

Biotype designation		Production of		
<i>H. influenzae</i>	<i>H. parainfluenzae</i>	Indole	Urease	Ornithine decarboxylase
I	V	+	+	+
II	VII	+	+	-
III	III	-	+	-
IV	II	-	+	+
V	VI	+	-	+
VI	I	-	-	+
VII	VIII	+	-	-
VIII	IV	-	-	-

Symbols: +, present; -, absent.

Table 3. Structures of *H. influenzae* capsular polysaccharides.<sup>a</sup>

Type	Structure
a	4)-β-D-Glc-(1 → 4)-D-ribitol-5-(PO <sub>4</sub> →
b	3)-β-D-Rib-(1 → 1)-D-ribitol-5-(PO <sub>4</sub> →
c	4)-β-D-GlcNAc-(1 → 3)-α-D-Gal-1-(PO <sub>4</sub> →
	3
	OAc
d	4)-β-D-GlcNAc-(1 → 3)-β-D-ManANAc-(1 →
	6
	R = serine, threonine, or alanine.
	R
e	3)-β-D-GlcNAc-(1 → 4)-β-D-ManANAc-(1 →
e'	3)-β-D-GlcNAc-(1 → 4)-β-D-ManANAc-(1 →
	3
	β-D-fructose
f	3)-β-D-GalNAc-(1 → 4)-α-D-GalNAc-1-(PO <sub>4</sub> →
	3
	OAc

Abbreviations: Glc, glucose; Gal, galactose; GlcNAc, *N*-acetyl glucosamine; and ManANAc, *N*-acetyl-D-mannosamine.

<sup>a</sup>Ribose and fructose are in the furanose ring form; Glc, Gal, GlcNAc, and ManANAc are in the pyranose ring form.

with encapsulated *H. influenzae* (Musser et al., 1986).

## Isolation and Identification

*Haemophilus* species are facultative anaerobes and have both a respiratory and a fermentative type of metabolism. They catabolize D-glucose and other carbohydrates, producing acids and sometimes gas. They are non-motile and non-spore-forming. In addition, they are oxidase positive and reduce nitrates to nitrites or beyond. For most species, growth is optimal at a temperature of 33–37°C in a humid environment.

*Haemophilus* species that require factor X are lacking the enzymes necessary for conversion of δ-aminolevulinic acid (ALA) to protoporphyrin IX, the penultimate intermediate in the heme biosynthesis pathway (Biberstein et al., 1963). In

most cases, the requirement for factor X can be satisfied by supplementation of culture media with purified protoporphyrin IX. An active ferrochelatase, which catalyzes ferrous iron insertion into the protoporphyrin nucleus to form heme, has been demonstrated in a panel of *H. influenzae* strains, and this enzyme is presumed to be present in any *Haemophilus* strain that is able to use protoporphyrin IX as the sole source of factor X (Loeb, 1995). Although few isolates depend on pre-formed heme for growth, typically blood or blood derivatives, such as crystalline hemin, are used as the source of factor X for cultivation of *Haemophilus*. Hemin requirements generally range from 0.1 to 10 µg/ml for *H. influenzae* (Biberstein and Spencer, 1962; Brumfitt, 1959; Evans et al., 1974; Gilder and Granick, 1947) but can be as high as 200 µg/ml for optimal growth of *H. ducreyi* (Hammond et al., 1978).

Determination of a requirement for factor X is easily accomplished using the porphyrin test (Kilian, 1974), in which a large loopful of bacteria is inoculated into 0.5 ml of 0.1 M sodium phosphate buffer, pH 6.9, containing 0.08 mM magnesium sulfate and 2 mM  $\delta$ -ALA and then incubated at 37°C for 4 hours. Factor X-independent bacteria incorporate the ALA into the heme biosynthesis pathway and excrete pathway intermediates, such as porphobilinogen and porphyrins. These intermediates emit a red fluorescence and can be detected by examination under a Wood's light (360 nm) in a dark room. Factor X-dependent bacteria lack the enzymes needed for production of these intermediates and do not excrete them.

The requirement for factor V is usually satisfied by supplementation of culture media with purified crystalline nicotinamide adenine dinucleotide (NAD), with alternatives including NAD phosphate (NADP) and nicotinamide mononucleoside. Typical NAD requirements range from 0.2 to 1  $\mu$ g/ml for *H. influenzae* and from 1 to 5  $\mu$ g/ml for *H. parainfluenzae*, although some strains exhibit requirements as high as 25  $\mu$ g/ml. When NAD-deficient agar media are used for the isolation of bacteria from sites of mixed microbial populations that include *Haemophilus*, small colonies of NAD-dependent *Haemophilus* organisms are often observed growing around larger colonies of other bacterial species, such as *Staphylococcus aureus* or *Pseudomonas aeruginosa*. This phenomenon is referred to as satellitism and arises from the ability of factor V-dependent *Haemophilus* colonies to utilize NAD secreted by other bacteria. In an application of this phenomenon, a plate previously streaked with *Haemophilus* is cross-inoculated with a single streak of the "feeder" (NAD-producing) organism. Growth of factor V-dependent *Haemophilus* strains is initially confined to the areas immediately adjacent to the feeder streak but eventually extends peripherally as the NAD diffuses through the agar. NAD-impregnated sterile paper disks can be used in place of cross-inoculation to demonstrate factor V-dependent satellitism.

Although the blood in sheep blood agar contains both NAD and hemin, NAD-degrading enzymes also present in the blood make this medium unsuitable for cultivation of most *Haemophilus* species. Instead, *Haemophilus* has historically been grown on chocolate agar, which is made by adding 5–10% defibrinated blood to sterilized blood agar base and holding the mixture at 80°C for 15 to 20 minutes, until the medium turns a dark brown color reminiscent of chocolate. This heating process both liberates NAD from the red blood cells and inactivates NAD-degrading enzymes, although it also results

in degradation of some of the NAD. A more uniform medium is obtained with "enriched" chocolate agar, in which factor V is provided as a constituent of a chemically defined supplement (either Isovitale X or supplement B) and factor X is provided from an artificial "hemoglobin" preparation consisting of washed and lysed red blood cells. A 2% solution of the "hemoglobin" preparation is autoclaved and added to an equal volume of sterilized double-strength GC medium base (a standard medium consisting of proteose peptone/corn starch/phosphate salts) after both solutions have cooled to 50°C. Isovitale X or supplement B is then added to a final concentration of 1% to complete the preparation.

Owing to its opacity, chocolate agar is less than optimal for certain applications, including visual screening of clinical specimens and laboratory strains for capsule production. Transparent plates made from supplemented brain heart infusion agar (BHIs agar) represent an alternative. Brain heart infusion agar is a commercially available rich medium that is autoclaved and then supplemented with a 1:1000 dilution of NAD stock solution (2 mg/ml  $\beta$ -NAD dissolved in water and filter sterilized) and one of several hemin sources, including purified crystalline hemin, clarified defibrinated horse blood, or Fildes extract (a commercially available peptic digest of blood). Another source of hemin is Levinthal's stock, which is prepared by boiling a mixture of 35% horse blood in autoclaved BHI broth and removing the particulate clotted matter by filtration through sterile filter paper or by centrifugation. Plates containing 2% Levinthal's stock traditionally contain a relatively high concentration of NAD, in the range of 10  $\mu$ g/ml. Clarified defibrinated horse blood and Fildes extract are generally preferred over Levinthal's stock because they are simpler to prepare. A solution of crystalline hemin is also relatively simple to prepare but results in plates with a shorter shelf life. Growth of *Haemophilus* in liquid culture is best accomplished in BHIs broth, which is prepared in the same manner as BHIs agar, except for omission of the agar.

Aside from the requirement for rich media supplemented with hemin and NAD, some *Haemophilus* species and occasional unusual isolates require special additional nutrients or alterations in growth conditions. For example, *H. ducreyi* grows well only on chocolate agar containing 5–10% fetal bovine serum (Nsanze et al., 1984; Sottnek et al., 1980) and grows optimally at a temperature of 33°C, compared to 35–37°C for other *Haemophilus* species (Sturm and Zanen, 1984). In addition, rare strains of *H. influenzae* require supplemental pantothenic acid, thiamine, uracil, or specific nucleotides for optimal growth (Evans and Smith, 1972; Holt, 1962). As

another example, it appears that increased CO<sub>2</sub> tension is a requirement for optimal growth and in some cases for any growth for many *Haemophilus* species, especially those whose names are derived from the Greek term “aphros,” referring to carbon dioxide bubbles produced by fermenting wine (*H. aphrophilus*, *H. paraphrophilus* and *H. paraphrohaemolyticus*). The metabolic basis for this supposed CO<sub>2</sub> dependence is not known, and in many of these organisms, an observed loss of CO<sub>2</sub> dependence upon subculture has cast doubt on the absolute nature of this requirement (Kilian, 1976; Zinnemann et al., 1971). One possibility is that the atmospheric effects of increased CO<sub>2</sub> tension actually serve to fulfill a growth requirement for increased ambient moisture, a hypothesis supported by the successful cultivation of several “CO<sub>2</sub>-dependent” strains of *H. influenzae* and *H. aphrophilus* in a moist environment lacking CO<sub>2</sub> (Hoiseith, 1992; Kraut et al., 1972). Nevertheless, a moist environment containing 5–10% CO<sub>2</sub> is generally recommended for cultivation of *Haemophilus* isolates (Albritton, 1989; Kilian, 1976).

## Preservation

Following growth on agar plates or in broth culture, *Haemophilus* strains remain viable for only a few days when stored at room temperature. *Haemophilus* species are not amenable to prolonged storage at 4°C either. Consequently, the preferred method for long-term storage involves deep freezing at temperatures below –70°C. Bacteria are prepared for deep freezing by swabbing growth from an agar plate into 2-ml cryovials containing brain heart infusion broth (or another suitable growth medium, such as heart infusion broth or Levinthal’s stock) supplemented with 10–20% sterile glycerol. Alternatively, broth cultures can be inoculated and grown to mid-log phase (OD<sub>600</sub> = 0.3–0.4), then transferred to cryovials containing glycerol, again aiming for a final concentration of 10–20% glycerol. As another option, strains can be stored in skim milk. Survival of *Haemophilus* at ambient temperatures is sufficient to allow short-term (1 to 3 days) transport of cultures either streaked onto chocolate agar or BHIs agar plates, which are then sealed with parafilm, streaked onto chocolate agar or BHIs agar slants, or inoculated into sealed plastic vials or glass ampoules containing BHIs broth or another suitable growth medium.

## Habitat and Ecology

*Haemophilus* species are members of the normal flora in the upper respiratory tract and occasion-

ally the genital area and gastrointestinal tract. *Haemophilus influenzae*, *H. parainfluenzae*, *H. haemolyticus*, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. aphrophilus*, *H. paraphrophilus* and *H. segnis* are found in the pharynx in healthy humans. *Haemophilus parainfluenzae*, *H. haemolyticus*, *H. parahaemolyticus*, *H. aphrophilus*, *H. paraphrophilus* and *H. segnis* are found in the oral cavity as well, and *H. aphrophilus*, *H. paraphrophilus*, *H. segnis* and *H. haemolyticus* also are often present in dental plaque and gingival scrapings. *Haemophilus influenzae* and *H. parainfluenzae* have occasionally been isolated from the urethra and vagina in women. *H. ducreyi* is primarily a genital pathogen in humans. Among animal species, *H. paragallinarum* is a member of the normal respiratory flora in fowl, *H. parasuis* is found in the respiratory tract of pigs, *H. paracuniculus* is present in the gastrointestinal tract of rabbits, and *H. haemoglobinophilus* forms part of the normal flora of the preputial sac of dogs. *Haemophilus somnus* occupies the respiratory tract and the genital tract of cattle, while the reservoir for *H. agni* is presumed to be a mucosal surface in sheep.

## Physiology

### Heme and Iron Acquisition

The requirement for heme in factor X-dependent *Haemophilus* species presents a challenge for the proliferation of these organisms during natural infection. Free heme is toxic to mammalian cells and is sequestered within the body by the carrier protein hemopexin. Hemoglobin, another usable source of heme in the human body, is sequestered by the carrier protein haptoglobin. To acquire heme from the host environment, factor X-dependent *Haemophilus* species express several multicomponent systems devoted to the efficient binding and uptake of heme from heme : hemopexin and hemoglobin : haptoglobin sources. Although these systems were first identified and characterized in several strains of *H. influenzae* type b, similar mechanisms have also been shown to exist in nontypeable *H. influenzae* and to some extent in *H. ducreyi*.

One system involved in binding heme : hemopexin complexes in *H. influenzae* consists of the proteins HxuA, HxuB and HxuC, which are all expressed from a single operon encompassing the *hxuA*, *hxuB* and *hxuC* genes (Cope et al., 1995; Hanson et al., 1992a). The 100-kDa HxuA protein is released from the bacterial surface into the culture supernatant, where it binds heme : hemopexin (Cope et al., 1998; Cope et al.,



1994). The 60-kDa HxuB protein mediates release of HxuA from the cell surface. The 78-kDa HxuC protein is thought to assist in transfer of bound heme into the bacterial cell because it is similar to TonB-dependent outer membrane proteins. TonB is an energy-transducing cytoplasmic membrane protein that facilitates a variety of transport processes via its interactions with members of a family of outer membrane proteins (Postle, 1990). The role of TonB in heme uptake and its importance in vivo were demonstrated by the inability of *tonB* mutants to utilize heme : hemopexin and by the reduced virulence of these mutants in an infant rat model of bacteremia (Jarosik et al., 1994). Mutation of either *tonB* or *hxuC* also results in an inability to grow on low levels of free heme, suggesting that these proteins are involved not only in the uptake of heme from heme:hemopexin complexes but also in the uptake of free heme (Cope et al., 1995; Jarosik et al., 1994). A second *H. influenzae* system involved in heme : hemopexin binding consists of a 57-kDa heme : hemopexin outer membrane receptor and two accessory proteins, including a 29-kDa outer membrane protein and the P2 outer membrane porin. Assembly of this complex is regulated by ambient levels of iron (Wong et al., 1994; Wong et al., 1995). Distinct heme binding properties in *H. influenzae* have also been associated with a 61-kDa lipoprotein encoded by *hbpA* and an iron-regulated 39.5-kDa outer membrane protein (Hanson et al., 1992b; Lee, 1992).

A number of different proteins capable of binding hemoglobin and hemoglobin : haptoglobin have been identified in *H. influenzae* (Frangipane et al., 1994). The first to be characterized was HgpA, a 120-kDa protein that is encoded by the *hgpA* gene and is expressed in response to conditions of limited heme availability (Jin et al., 1996; Jin et al., 1999). HhuA, a 115-kDa homologue of HgpA identified in a different *H. influenzae* strain, has also been shown to bind hemoglobin:hemopexin (Maciver et al., 1996). Like HxuC, HgpA and HhuA exhibit similarity to the TonB-dependent family of outer membrane proteins. Interestingly, mutation of *hgpA* in *H. influenzae* strain HI689 did not restrict growth with hemoglobin or hemoglobin : haptoglobin, and subsequent characterization of this mutant led to the discovery of two additional hemoglobin-binding proteins encoded by separate genes, designated *hgpB* and *hgpC* (Morton et al., 1999; Ren et al., 1998). These proteins are 115 kDa and 120 kDa, respectively, and both are highly homologous to HgpA. Expression of either HgpA, HgpB or HgpC is both necessary and sufficient to support growth with hemoglobin or hemoglobin:haptoglobin. Further analysis of the genes encoding these proteins in several

different strains revealed the presence of CCAA nucleotide repeats in the 5'-coding sequence, suggesting that expression of these proteins is subject to phase variation by a slipped-strand mispairing mechanism (Cope et al., 2000; Ren et al., 1999). Given the high degree of sequence similarity between HgpA, HgpB, HgpC and HhuA, it is likely that these proteins represent homologues that are expressed in different strains and have been duplicated in some instances. Of note, a homologue of HgpA has been identified in *H. ducreyi* strain 35000, and expression of this hemoglobin-binding protein is critical for virulence in human volunteer studies (Al-Tawfiq et al., 2000; Elkins, 1995a; Elkins et al., 1995b).

Similar to the situation with heme, elemental iron is toxic to mammalian cells and is therefore sequestered by the host. Proteins that sequester iron include transferrin, lactoferrin and ferritin. Levels of free iron in the body are maintained far below those required for survival and growth of microorganisms (Otto et al., 1992). Unlike a number of other bacteria, *Haemophilus* species have not been reported to secrete siderophores, soluble proteins capable of binding free iron or iron complexed with host proteins (Morton and Williams, 1990; Weinberg, 1978). Instead, many *Haemophilus* species rely on a complex system of proteins that engage in specific binding of transferrin at the bacterial cell surface, removal of iron from transferrin, and transport of free iron into the cell (Hardie et al., 1993; Herrington and Sparling, 1985; Schryvers, 1988; Schryvers, 1989; Schryvers and Gray-Owen, 1992). As with the systems for heme uptake, for the most part, the proteins involved in iron acquisition by *Haemophilus* were identified first in *H. influenzae*, including type b and nontypeable strains. Interestingly, *H. influenzae* can bind transferrins from human, bovine and rabbit sources but not from several other mammalian species (Herrington and Sparling, 1985; Morton and Williams, 1990; Schryvers, 1988; Schryvers, 1989), perhaps accounting in part for the host specificity of this organism. Similarly, the bovine pathogen *H. somnus* binds bovine transferrin selectively, and the porcine pathogen *H. parasuis* binds porcine transferrin preferentially (Charland et al., 1995; Ogunnariwo et al., 1990).

During the process of *H. influenzae* acquisition of iron from transferrin, two outer membrane proteins are responsible for binding transferrin at the bacterial cell surface. Transferrin-binding protein 1 (Tbp1) is a 95-kDa integral membrane protein encoded by *tbpA*, and Tbp2 is a lipid-anchored variable size protein, ranging from 68 to 85 kDa, encoded by *tbpB* (Gray-Owen and Schryvers, 1995a; Gray-Owen et al., 1995b; Stevenson et al., 1992). Both Tbp1 and

Tbp2 are capable of binding transferrin and are expressed as a single transcriptional unit under the control of a ferric uptake regulatory (Fur-like) repressor protein (Gray-Owen and Schryvers, 1995a). Expression of Tbp1 and Tbp2 in vivo is supported by RT-PCR analysis of samples from patients with otitis media (Whitby et al., 1997) and by reactivity of these proteins with sera obtained from healthy adults (Holland et al., 1992; Holland et al., 1996). Based on the predicted amino acid sequence, Tbp1 is presumed to be a TonB-dependent protein, a conclusion supported by the observation that inactivation of *tonB* eliminates the ability of *H. influenzae* to utilize iron from transferrin (Jarosik et al., 1995). Although mutation of either *tbpA* alone or both *tbpA* and *tbpB* together eliminates utilization of iron from transferrin, mutation of *tbpB* alone is permissive for the utilization of transferrin, provided transferrin is present in high concentration (Gray-Owen and Schryvers, 1995a). With this information in mind, one hypothesis is that Tbp2 extends from the bacterial surface and binds transferrin, thereby increasing the effective concentration of transferrin at the cell surface and facilitating interaction with Tbp1. Tbp1, in turn, initiates the TonB-dependent release of iron and the transport of free iron into the periplasm (Rao et al., 1999).

Subsequent transfer of iron from the periplasmic space into the cytoplasm relies on proteins encoded by the *hitA*, *hitB* and *hitC* genes, which are located on a single 4-kb operon regulated by a Fur-like repressor (Adhikari et al., 1995; Sanders et al., 1994). The *hitA* gene encodes FbpA, a periplasmic high-affinity iron-chelating protein that belongs to the transferrin superfamily and is essential for iron uptake (Bruns et al., 1997; Kirby et al., 1997). The *hitB* gene encodes a cytoplasmic membrane permease (HitB), and *hitC* encodes a nucleotide binding protein (HitC). Presumably, free iron in the periplasm is bound by FbpA and transferred to HitB, which then actively transports the iron into the cytoplasm in a step energized by HitC.

### Sugar Metabolism

In light of the small genomes and fastidious growth requirements of *Haemophilus* species, it is not surprising that these organisms employ relatively simple pathways for sugar metabolism. Based on biochemical and genetic information, *Haemophilus* species appear to utilize the Embden-Meyerhof-Parnas, the hexose monophosphate and the Entner-Duodoroff pathways, and the tricarboxylic acid cycle (Fleischmann et al., 1995; Holländer, 1976a; Klein, 1940; White, 1966). Glycolytic metabolism in the absence of

an oxidative electron transport chain has been observed only in selected factor X-dependent species of *Haemophilus*, including *H. influenzae* and *H. haemoglobinophilus* (White, 1963). Extensive biochemical analysis of one strain of *H. parainfluenzae* suggests that in species capable of oxidative sugar catabolism, glycolysis occurs only in the presence of a terminal electron acceptor, such as oxygen, nitrate or fumarate (White, 1966; White and Sinclair, 1971). Electron transfer in these species proceeds from flavoproteins via cytochromes *b*, *c*, *d* and *a* to demethylmenaquinone (DMK), to cytochrome *o*, and finally to an oxygen or nitrate terminal electron acceptor (Holländer, 1976b; White and Sinclair, 1971). Most *Haemophilus* species appear to use DMK as the sole respiratory quinone. In *H. haemolyticus*, *H. haemoglobinophilus*, *H. parvus* and *H. paragallinarum*, ubiquinone is present as well (Holländer et al., 1981; Holländer and Manheim, 1975).

In *H. influenzae*, fructose appears to play a central role in sugar metabolism. Fructose uptake by *H. influenzae* occurs via a phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) that phosphorylates fructose as it is transported across the cytoplasmic membrane. In other Gram-negative bacteria, the PTS serves as both transport machinery and sensor for a number of preferred sugar substrates (Postma et al., 1993). Limitations in the availability of these preferred sugars results in a backup of the phosphotransferase enzymes, which initiates increased cAMP production by adenylate cyclase and subsequent cAMP-receptor protein (CRP)-mediated transcriptional activation of genes involved in the uptake and catabolism of other sugar substrates (Botsford and Harman, 1992). Genetic and biochemical analysis has demonstrated that the PTS of *H. influenzae* is specific for fructose, unlike the situation with other Gram-negative organisms (Macfadyen et al., 1996b). In the absence of available fructose, *H. influenzae* upregulates transport and catabolism of galactose, ribose, xylose and fucose. Additionally, *H. influenzae* imports and catabolizes glycerol, glucose and sialic acid in a PTS-independent manner.

Several facets of *H. influenzae* sugar metabolism deserve further comment. In particular, at first glance the central role of fructose is difficult to understand given the limited availability of this sugar in the human upper respiratory tract, the usual habitat of *H. influenzae*. However, it is possible that a preference for fructose serves *H. influenzae* in two separate ways: first, it may prevent *H. influenzae* from having to compete for glucose with host cells and other commensal bacteria; second, it may be advantageous to rapid proliferation of bacteria during invasion of the

cerebrospinal fluid, where fructose concentrations are 30-fold higher than serum levels (Macfadyen and Redfield, 1996a). In addition, it is interesting that *H. influenzae* utilizes sialic acid. This sugar is readily available in the respiratory tract as a terminal component of branched chain oligosaccharides on glycoproteins found on the surfaces of epithelial cells, in mucus and in serum. Although *H. influenzae* possesses many of the enzymes necessary for uptake and catabolism of sialic acid (Casari et al., 1995; Vimr et al., 2000), a gene encoding the neuraminidase necessary for liberating sialic acid from oligosaccharides is conspicuously absent from the *H. influenzae* Rd genome. However, neuraminidase activity has been reported in selected *H. influenzae* isolates (Muller and Hinz, 1977), and strains that lack a neuraminidase may be dependent on the neuraminidase activity of other respiratory commensal organisms or pathogens, such as *Pseudomonas aeruginosa* (Macfadyen and Redfield, 1996a). Neuraminidase activity is also seemingly absent in *H. somnus* and *H. aphrophilus* but has been demonstrated in *H. parasuis* and *H. paragallinarum* (Hinz and Muller, 1977; Lichtensteiger and Vimr, 1997). Of further note, *H. influenzae* appears to express a constitutively active nucleoside uptake system and a PTS-dependent competence induction system. Thus, natural competence may serve a second, non-genetic purpose of providing bacteria with an energetically efficient source of nucleotides from a mucosal environment rich in DNA (Lethem et al., 1990; Matthews et al., 1963) but limited in energy substrates required during de novo nucleotide synthesis (Macfadyen and Redfield, 1996a).

## Genetics

*Haemophilus influenzae* is a naturally transformable organism and readily takes up DNA from its environment. Transformation of this organism was observed as early as 1951 when Alexander and Leidy reported that capsule production could be restored in nonencapsulated R variants of each of the six *H. influenzae* capsular serotypes after exposure to DNA isolated from encapsulated donor strains (Alexander and Leidy, 1951). Additional studies demonstrated that strain Rd (derived from a serotype d strain) was most efficient among the tested strains at transformation with DNA from representatives of all capsular serotypes (Alexander et al., 1954), and this strain has since become the workhorse of *Haemophilus* genetic analysis and manipulation. Such efforts have been greatly enhanced by the publication of the 1.83-Mb Rd genome in 1995 (Fleischmann et al.,

1995), the very first genome of a cellular organism to be sequenced in its entirety. The Rd genome and emerging sequence information for the genome of *H. ducreyi* strain 35000 can be accessed through the website of {The Institute for Genomic Research}.

Although mechanisms and applications of genetic manipulation are most extensively described for *H. influenzae* strain Rd, transformation has been used with great success for manipulation of clinical isolates of both encapsulated and nontypeable *H. influenzae* and other *Haemophilus* species as well, including *H. parainfluenzae*, *H. ducreyi*, *H. parasuis*, *H. paragallinarum* and *H. somnus* (Gonzales et al., 1996; Gromkova and Goodgal, 1979; Sanders et al., 1997; Stevens et al., 1995). As with the *H. influenzae* R variants, variation in transformation efficiency exists both across and within *Haemophilus* species. For example, a recent study examining transformation efficiencies of clinical isolates of *H. parainfluenzae* found that biotype II strains were highly transformable, while biotype I and biotype III strains were relatively refractory to transformation (Gromkova et al., 1998). Similarly, transformation efficiency varies considerably from one strain of *H. influenzae* to another, with some strains being nontransformable (Rowji et al., 1989).

Early work demonstrated successful transformation of a number of *Haemophilus* species with both intraspecific and interspecific *Haemophilus* DNA (Beattie and Setlow, 1970; Leidy et al., 1959; Leidy et al., 1956; Leidy et al., 1965; Schaeffer, 1958; Steinhardt and Herriott, 1968; White et al., 1964). Subsequent studies showed that transformation of *Haemophilus* species occurs much more efficiently with DNA from *Haemophilus* than with DNA from bacteria of other genera (Danner et al., 1982b; Goodgal and Mitchell, 1984; Kahn and Smith, 1984; Mathis and Scocca, 1982; Scocca et al., 1974; Sisco and Smith, 1979). In the case of *H. influenzae*, this preference is a consequence of the mechanism of DNA uptake by naturally competent organisms, a process that involves specific recognition of a nine base pair sequence (AAGTGCGGT) within the transforming DNA by receptors present on the bacterial cell surface (Deich and Smith, 1980). This uptake sequence is common throughout the *H. influenzae* chromosome but is generally rare or absent in non-*Haemophilus* DNA. In the 1.83-Mb genome of *H. influenzae* strain Rd, the uptake sequence occurs at 1,465 sites, with an average distance of 1,224 bp between two such sites (Smith et al., 1999). Interestingly, the sites occur more often in intergenic regions of the chromosome than in coding sequence, and the existence of multiple pairs of uptake sequences in inverted repeat orientations suggests that

these pairs may form stem-loop structures that act as transcriptional terminators.

The cellular receptors to which *Haemophilus* DNA uptake sequences bind are components of membranous blebs termed “transformasomes,” which appear on the cell surface during induction of competency (Barany et al., 1983; Kahn et al., 1983). After binding to *H. influenzae* transformasomes, DNA passes into the bacterial cell via a process that results in the complete degradation of one DNA strand and partial degradation of the other strand, termed the “entering strand” (Barany et al., 1983). The bacterial chromosome is somehow accessible to DNA within the transformasome, and degradation of the entering strand ceases once it finds a homologous site within the chromosome and becomes incorporated by *rec*-dependent recombination. DNA entry into *H. parainfluenzae* cells involves a somewhat different set of events with far less degradation of DNA within the transformasome (Barany and Kahn, 1985). Transformation of *Haemophilus* with stable plasmids is thought to depend on the low frequency accidental escape of plasmid DNA from the transformasome (Pifer, 1986). Maintenance of a stable plasmid is also dependent on the absence of recombination between plasmid and chromosomal DNA, and for this reason, recombination-deficient strains, such as the DB117 *recA* isogenic mutant of *H. influenzae* Rd, are usually used as recipients for transformation of plasmids.

Although *Haemophilus* is naturally competent, only about one cell in ten thousand is capable of DNA uptake during exponential growth. However, competence can be induced in an entire population by conditions of slowed growth that occur during nutrient deprivation or transient anaerobiosis, leading to transformation efficiencies as high as 1–5%. The mechanisms that underlie competence induction, DNA uptake, and DNA integration in *Haemophilus* constitute a genetically programmed response to such growth conditions. These mechanisms have been studied most extensively in *H. influenzae* strain Rd. Competence induction in response to nutrient deprivation is dependent on a functioning fructose-specific phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), a multi-enzyme component of the bacterial sugar import machinery (Gwinn et al., 1996; Macfadyen et al., 1996b). Sensing of sugar availability by the PTS acts in concert with a cellular cAMP phosphodiesterase to modulate levels of cellular cAMP, which presumably regulates expression of competence-associated genes via cAMP receptor protein (CRP)-mediated transcriptional activation (Macfadyen et al., 1998). Examination of mutant strains defective in competence induction or DNA uptake has identified a number of

essential genes involved in these processes. These include genes encoding a periplasmic disulfide oxidoreductase (*por*) homologous to *dsbA* of *Escherichia coli*, a type IV pilin-like protein biogenesis operon, several drug efflux transporters, a phospholipid biosynthesis enzyme, and several loci encoding genes of unknown function (Beattie and Setlow, 1971; Dougherty and Smith, 1999; Gwinn et al., 1998; Larson and Goodgal, 1991; Redfield, 1991; Tomb, 1992; Tomb et al., 1989).

Over the years, several methods have been developed to transform *Haemophilus* (Goodgal and Herriott, 1961; Herriott et al., 1970; Stuy, 1962). In experiments with *H. influenzae*, the M-IV method is most efficient and involves incubation of early log-phase bacteria in defined minimal medium, then exposure to transforming DNA, and then overnight outgrowth on selective medium (see Barcak et al., 1991 for details). This protocol results in transformation rates of  $\sim 10^7$  transformants per ml of competent cells exposed to 1  $\mu\text{g}$  of chromosomal DNA. A second method developed in *H. influenzae* involves overnight growth of bacteria in a small volume of a complex liquid medium that contains transforming DNA, then selection on solid medium (see Barcak et al., 1991 for details). The growth conditions used to achieve natural transformation in *H. influenzae* result in a substantial loss of viability when applied to *H. parainfluenzae* (Barany and Kahn, 1985). As a consequence, *H. parainfluenzae* is transformed instead by a method that involves overnight incubation of a small volume of bacteria in a stationary Petri dish (Gromkova and Goodgal, 1979). Efficient transformation of *H. influenzae*, *H. ducreyi* and *H. somnus* has been accomplished by methods developed for enteric bacteria, including electroporation (Dower et al., 1988) and the calcium chloride ( $\text{CaCl}_2$ ) method (Barcak et al., 1991; Sanders et al., 1997; Stevens et al., 1995).

Owing to the complex nutritional requirements of *Haemophilus* species, auxotrophic markers have not been used for selection of transformants. On the other hand, antibiotic resistance markers have been used extensively. A number of plasmid cloning vectors incorporating a variety of antibiotic resistance genes have been constructed for the purpose of *Haemophilus* genetic manipulation. Many of these plasmids are able to serve as shuttle vectors between *Haemophilus* and *E. coli* because they contain origins of replication for both organisms (Table 4). The first such vector to be described was pHVT1, which carries the *Haemophilus* pRSF0885 origin of replication (*ori*) along with ampicillin and tetracycline resistance genes (Danner and Pifer, 1982a). This plasmid has spawned a number of derivatives containing

Table 4. *H. influenzae* cloning vectors.

Vector	<i>H. influenzae</i> plasmid origin	<i>E. coli</i> plasmid origin	Size (kb)	Selectable markers	Unique restriction sites
pHVT1	pRSF0885 <sup>a</sup>	pMB1	10.9	Ap, Tc	<i>Pst</i> I, <i>Cla</i> I, <i>Eco</i> RI, <i>Mlu</i> I, <i>Eco</i> RV, <i>Nco</i> I
pGJB103	pRSF0885	pMB1	8.7	Ap, Tc	<i>Pst</i> I, <i>Cla</i> I, <i>Eco</i> RI, <i>Mlu</i> I, <i>Eco</i> RV, <i>Nco</i> I
pHJ1	pRSF0885	pMB1	7.0	Ap, Tc	<i>Pst</i> I, <i>Cla</i> I, <i>Eco</i> RI, <i>Mlu</i> I, <i>Eco</i> RV, <i>Nco</i> I
pHK1	pRSF0885	pMB1	9.8	Km, Tc	<i>Eco</i> RI, <i>Eco</i> RV, <i>Nco</i> I, <i>Nru</i> I, <i>Sma</i> I, <i>Xho</i> I
pVT63	pRSF0885	pMB1	6.4	Ap, Km	<i>Hinc</i> II, <i>Pst</i> I, <i>Sca</i> I, <i>Cla</i> I, <i>Hind</i> III, <i>Nru</i> I, <i>Sma</i> I, <i>Xho</i> I
pVT64	pRSF0885	pMB1	6.9	Ap, Sp	<i>Hinc</i> II, <i>Pst</i> I, <i>Sca</i> I, <i>Ssp</i> I
pVT65	pRSF0885	pMB1	6.7	Ap, Cm	<i>Hinc</i> II, <i>Pst</i> I, <i>Sca</i> I, <i>Ssp</i> I, <i>Pvu</i> I
pVT66	pRSF0885	pMB1	7.2	Ap, Cm	<i>Hinc</i> II, <i>Pst</i> I, <i>Sca</i> I, <i>Sma</i> I, <i>Pvu</i> I
pDM2	pRSF0885	pMB1	9.8	Ap, Cm	<i>Bgl</i> II, <i>Pst</i> I, <i>Pvu</i> I, <i>Sma</i> I
pAT4	pRI234/pJS1867	–	8.0	Ap, Tc	<i>Pst</i> I, <i>Eco</i> RI
pACYC177	– <sup>b</sup>	p15A	3.9	Ap, Km	<i>Hind</i> II, <i>Hind</i> III, <i>Pst</i> I, <i>Xho</i> I, <i>Nru</i> I, <i>Sma</i> I, <i>Bam</i> HI
pACYC184	–	p15A	4.2	Cm, Tc	<i>Eco</i> RI, <i>Hind</i> III, <i>Bam</i> HI, <i>Sal</i> I
pFP10	– <sup>c</sup>	– <sup>c</sup>	5.4	Cm	<i>Hind</i> III, <i>Pst</i> I
pLS88	– <sup>d</sup>	– <sup>d</sup>	4.8	Km, Sm, Su	<i>Pst</i> I, <i>Cla</i> I, <i>Hind</i> III, <i>Pvu</i> I, <i>Sma</i> I, <i>Xho</i> I

Abbreviations and working concentrations ( $\mu\text{g/ml}$ ) for selectable markers in *H. influenzae*: Ap, Ampicillin (2–10); Cm, Chloramphenicol (2); Km, Kanamycin (25–50); Sm, Streptomycin (250); Sp, Spectinomycin (15); Su, sulfonamides; Tc, Tetracycline (5–10).

<sup>a</sup>Plasmids carrying only the pRSF0885 ori are capable of replication in both *H. influenzae* and *E. coli*.

<sup>b</sup>The p15A ori is capable of directing replication in both *E. coli* and *H. influenzae*.

<sup>c</sup>The *N. gonorrhoeae* pJD5 ori of pFP10 is capable of directing replication in *H. influenzae*, *H. ducreyi*, and *E. coli*.

<sup>d</sup>pLS88 was isolated from *H. ducreyi* and is also capable of replicating in *H. influenzae* and *E. coli*.

additional unique restriction sites and other antibiotic resistance markers (Barcak et al., 1991). Additional cloning vectors have been described that also use the pRSF0885 ori but contain either different antibiotic selection markers or different *E. coli* origins of replication (McCarthy et al., 1982; Trieu and McCarthy, 1990). Several *Haemophilus* cloning vectors containing origins of replication distinct from that of pRSF0885 have also been described. One example is pLS88, which was originally isolated from *H. ducreyi*. This plasmid carries resistance markers for sulfonamides, streptomycin and kanamycin (Dixon et al., 1994; Willson et al., 1989). Plasmids carrying the *E. coli* P15A replication origin, including pQL1, pACYC184, pACYC177, pSU2718 and pSU2719, are also able to replicate in *Haemophilus* (Barcak et al., 1991; Heidecker et al., 1994). Recently Pagotto et al. described a panel of vectors that contain a *Neisseria gonorrhoeae* ori and are capable of replicating in *Neisseria*, *Haemophilus* and *E. coli* (Pagotto et al., 2000). In addition to the introduction of stable plasmids and site-directed mutagenesis by insertional inactivation, transformation of various transposon elements conferring kanamycin resistance into *H. influenzae*, *H. ducreyi* and *H. paragallinarum* has been successfully employed for random insertion mutagenesis (Gonzales et al., 1996; Gwinn et al., 1997; Kauc and Goodgal, 1989; Kraiss et al., 1998; Stevens et al., 1995).

In contrast to the ease of transformation, other methods of genetic manipulation such as phage transduction and conjugation have not

been well established in *Haemophilus*. Four different bacteriophages have been identified in isolates of *H. influenzae*, although none appears to be useful for transduction (Boling et al., 1973; Stuy, 1978). Conjugation pili have not been identified in any *Haemophilus* species, and low frequency conjugal transfer of chromosomal genes by *H. influenzae* has been reported only once, in a system that utilized an *E. coli* F' plasmid (Deich and Green, 1987). Thus, transformation remains the mainstay of *Haemophilus* genetic manipulation.

## Epidemiology and Pathogenesis of Disease

### *H. influenzae*

Historically, *H. influenzae* type b has accounted for over 95% of invasive *H. influenzae* disease. A combination of non-type b encapsulated strains and nontypeable strains have accounted for the remaining cases. Non-type b encapsulated strains are especially important in developing countries. As an example, among children in Papua New Guinea, approximately 25% of lower respiratory tract isolates and 15% of cerebrospinal fluid (CSF) isolates of *H. influenzae* are non-type b encapsulated strains, especially type a and type f (Gratten and Montgomery, 1991; Lehmann, 1992).

Before the implementation of routine immunization against *H. influenzae* type b in devel-

oped countries, this organism was the leading cause of bacterial meningitis and epiglottitis in children younger than 5 years, accounting for 10,000 to 12,000 cases per year in the United States in the mid-1980s. *Haemophilus influenzae* type b was also a major cause of septic arthritis, pneumonia, pericarditis and facial cellulitis among young children. In the 1980s, approximately 1 in 200 children in the United States experienced invasive *H. influenzae* type b disease before the age of 5 years, with the peak incidence occurring between six months and two years of age (Cochi et al., 1985). Disease was most prevalent in boys, African Americans, Alaskan Eskimos, Apache and Navajo Indians, day care center attendees, and children living in overcrowded conditions. Pre-existing medical conditions such as sickle cell anemia, asplenia, human immunodeficiency virus (HIV) infection, certain immunodeficiency syndromes, and malignancies were important predisposing factors for *H. influenzae* type b systemic infection. Over the past 10 years, the incidence of invasive *H. influenzae* type b disease in developed countries has fallen dramatically (Rosenstein and Perkins, 2000). This decline has occurred even in regions where rates of immunization against *H. influenzae* type b are well below 100%, reflecting unexpected herd immunity related to decreased rates of carriage (Centers for Disease Control and Prevention, 1998a). Currently, only 100 to 300 cases of invasive *H. influenzae* type b disease are reported in the United States each year, mostly in non-vaccinated or under-vaccinated children, particularly in Native American communities where carriage rates remain high (Bisgard et al., 1998; Galil et al., 1999).

Despite the successful reduction of invasive *H. influenzae* type b disease in industrialized countries, *H. influenzae* type b remains an important pathogen in developing countries, where routine immunizations are still not available to most of the population. In these countries, *H. influenzae* type b remains the leading cause of bacterial meningitis and the second leading cause of bacterial pneumonia, resulting in up to 500,000 deaths per year in children younger than five years of age (Levine et al., 1998). A recent meta-analysis of *H. influenzae* type b-related medical reports from 75 countries estimated that vaccine usage by a total of 38 countries has resulted in just a 5.7% worldwide decrease in *H. influenzae* type b-related meningitis and less than a 2% worldwide decrease in all *H. influenzae* type b-related diseases, including pneumonia (Peltola, 2000). These figures reflect the current lack of vaccine availability in approximately 175 countries, most notably populous nations in Asia and Africa, where upwards of 118 million children remain unvaccinated (Children's Vaccine Initiative, 1999).

Nontypeable *H. influenzae* is present in the upper respiratory tract of 40–80% of healthy children and adults (Ingvarson et al., 1982; Kilian et al., 1972; Kuklinska and Kilian, 1984). Although nontypeable strains usually exist as commensal organisms in the nasopharynx, they are also an important cause of localized respiratory tract disease. In children, these organisms are the most common cause of purulent conjunctivitis, the second most common cause of otitis media (after *Streptococcus pneumoniae*), a frequent cause of acute and chronic sinusitis, and an important cause of exacerbations of cystic fibrosis (Gilligan, 1991). Among children in developing countries, nontypeable strains are a common cause of pneumonia and a significant source of mortality (Monto, 1989). In adults, nontypeable *H. influenzae* is a common cause of community-acquired pneumonia, otitis media, sinusitis, and exacerbations of underlying lung disease, including chronic bronchitis and bronchiectasis (Kilbourn et al., 1983).

Nontypeable *H. influenzae* is also an occasional cause of serious systemic disease such as septicemia, endocarditis, meningitis and septic arthritis. In individuals with bacteremia, abnormalities in humoral immunity are especially common, with examples including hypogammaglobulinemia, acquired immunodeficiency syndrome (AIDS), lymphoproliferative disease and acute leukemia. Meningitis accounts for a high percentage of nontypeable *H. influenzae* invasive disease, especially in children under 5 years of age. In most cases, meningitis develops as a complication of a CSF leak or by direct extension from a contiguous focus of infection, such as otitis media or sinusitis. During the past two decades nontypeable *H. influenzae* has become recognized as a cause of early-onset neonatal sepsis that occurs most often in premature infants and is acquired from the maternal urogenital tract (Friesen and Cho, 1986; Stull et al., 1988). Several studies indicate that many of these neonatal isolates are members of a cryptic biotype IV genospecies that is not common at other sites of infection and appears to be a genetically distinct *Haemophilus* species (Quentin et al., 1989; Quentin et al., 1996).

Because of the worldwide prevalence of *H. influenzae* disease, this organism has been the subject of intense laboratory investigation. A wealth of information relating to the molecular mechanisms of *H. influenzae* disease pathogenesis has emerged, and these mechanisms are described in detail in a number of excellent review articles (Foxwell et al., 1998; Moxon, 1992; Murphy, 2000; Rao et al., 1999; St. Geme, 1997). Several animal models of *H. influenzae* disease exist, including rat and chinchilla models of otitis media, a mouse pulmonary clearance

model, and an infant rat model of bacteremia and meningitis (Green et al., 1994; Hansen and Toews, 1992; Moxon et al., 1977; Yang et al., 1998a). However, these models fail to reproduce all aspects of natural disease, reflecting the fact that *H. influenzae* is a human-specific pathogen. Accordingly, much of our knowledge about interactions between *H. influenzae* and the human host has come from in vitro studies employing human adenoid and nasal turbinate tissue in organ culture, primary human respiratory epithelial cells, and cultured human cell lines.

Spread of *H. influenzae* occurs via inhalation of airborne droplets or by direct contact with respiratory tract secretions from an infected individual. Colonization of the new host is facilitated by pilus and non-pilus adhesins, which promote intimate interaction between bacteria and the respiratory mucosa, including respiratory epithelium and mucus. Both type b and selected nontypeable strains express hemagglutinating pili, which were first identified based on their ability to mediate bacterial attachment to human oropharyngeal epithelial cells and agglutination of human erythrocytes (Guerina et al., 1982; Pichichero et al., 1982; Stull et al., 1984). The proteins responsible for pilus biogenesis are encoded by a cluster of five genes designated *hifA-hifE* and include a major structural subunit (HifA), a periplasmic chaperone (HifB), an outer membrane usher (HifC), a nucleating structural subunit (HifD), and a pilus tip adhesin (HifE) (Rao et al., 1999). *Haemophilus influenzae* pili probably interact with sialyllactosylceramide on host epithelial cells, as compounds containing this structure are able to inhibit both hemagglutination and pilus-mediated attachment to epithelial cells (van Alphen et al., 1991). In addition, pili have been shown to promote bacterial adherence to respiratory mucins and to the heparin-binding domain of fibronectin (Kubiet et al., 2000; Virkola et al., 2000).

Approximately 75–80% of nontypeable strains express high-molecular-weight non-pilus adhesins referred to as HMW1 and HMW2, which are highly related to each other and share homology with filamentous hemagglutinin, an adhesin and colonization factor expressed by *Bordetella pertussis* (Barenkamp and Leininger, 1992; St. Geme et al., 1998). Overall, the predicted amino acid sequences of HMW1 and HMW2 are 71% identical and 80% similar. Despite their homology, these proteins exhibit different cellular binding specificities, suggesting that they interact with distinct receptor structures and may serve complementary functions in the process of colonization (Dawid et al., 2001; Hultgren et al., 1993). Experiments with HMW1 have demonstrated that this adhesin recognizes

a glycoprotein receptor containing N-linked oligosaccharide chains with sialic acid in an  $\alpha$ -2-3 configuration (St. Geme, 1994a). At this point, the HMW2 receptor remains poorly characterized. Expression of both HMW1 and HMW2 is controlled by the number of 7-bp repeats present in the promoter region of the structural gene, with the number of repeats varying via slipped-strand mispairing and influencing transcription (Dawid et al., 1999).

Among nontypeable strains that lack HMW adhesins, almost all express an alternative non-pilus adhesin called Hia (Barenkamp and St. Geme, 1996b; St. Geme et al., 1998). The Hia protein is a member of the growing family of autotransporter proteins, which includes the *N. gonorrhoeae* and *H. influenzae* IgA1 proteases and a number of other Gram-negative bacterial proteins implicated in virulence (Henderson et al., 1998). Autotransporters employ a distinctive mechanism of secretion, with the C-terminal domain of the protein forming a  $\beta$ -barrel porin in the outer membrane and facilitating translocation of the rest of the protein, called the passenger domain, to the cell surface. *Haemophilus influenzae* type b strains express a ~240-kDa homologue of Hia called Hsf. In experiments with cultured epithelial cells, Hia and Hsf confer the same adhesive specificities, suggesting that the *hia* and *hsf* genes are alleles of the same locus (St. Geme et al., 1996). Overall, Hia and Hsf share 72% identity and 80% similarity. The two proteins are most similar at their N-terminal and C-terminal ends and also contain a conserved internal domain that is repeated three times in Hsf, thus accounting in part for the difference in size (St. Geme et al., 1996). One hypothesis is that the larger size of Hsf reflects the need for the adhesive domain to extend beyond the confines of the type b capsule.

Nearly all nontypeable *H. influenzae* strains express a 155-kDa protein called Hap which was first discovered based on its ability to promote bacterial entry into epithelial cells (St. Geme et al., 1994b). In addition, Hap promotes adherence to a variety of cultured epithelial cells (St. Geme et al., 1994b). Like Hia, Hap is an autotransporter protein and consists of a C-terminal  $\beta$ -barrel domain (called Hap $\beta$ ) and an internal passenger domain (called Hap $_s$ ). Hap $_s$  contains the adhesive activity of the protein and also possesses serine protease activity that mediates the autoproteolytic cleavage of Hap $_s$  from Hap $\beta$  and subsequent release of Hap $_s$  from the cell surface (Hendrixson et al., 1997). Of interest, interactions between Hap $_s$  molecules retained on the cell surface result in bacterial aggregation and microcolony formation. Hap autoproteolytic activity is blocked by secretory leukocyte protease inhibitor (SLPI), a compound that is

present in normal respiratory secretions and is upregulated during states of inflammation (Hendrixson and St. Geme, 1998). One hypothesis is that local concentrations of SLPI may modulate the amount of Hap<sub>s</sub> retained on the cell surface, thus influencing patterns and levels of bacterial association with respiratory epithelial tissue and spread of bacteria through the respiratory tract. As another possibility, the serine protease activity of Hap<sub>s</sub> may be important for degradation of host proteins involved in structural integrity of respiratory epithelium or the immune response.

In recent work, several additional adhesive activities have been identified in *H. influenzae*. Using thin layer chromatography, Busse et al. found that type b and nontypeable strains are capable of recognizing phosphatidylethanolamine (PE), gangliotriosylceramide (Gg3), gangliotetrosylceramide (Gg4), sulfatoxygalactosylceramide, and, to a lesser extent, sulfatoxygalactosylglycerol (Busse et al., 1997). To extend this observation, these investigators prepared a phosphatidylethanolamine affinity matrix and purified a 46-kDa protein that inhibits binding of whole bacteria to immobilized PE and Gg3. More recently, Hartmann and Lingwood reported that heat shock treatment resulted in a marked increase in binding by nontypeable *H. influenzae* to sulfatoxygalactosylceramide (Hartmann and Lingwood, 1997). Additional analysis suggested that this binding is due to two Hsp70-related heat shock proteins. In studies with respiratory tract mucins, Reddy and co-workers reported that outer membrane proteins called P2 and P5 promote appreciable binding (Reddy et al., 1996). Lipooligosaccharide (LOS) is another molecule that influences interactions with host cells, recognizing platelet activating factor (PAF) receptor and facilitating both adherence and invasion (Swords et al., 2000). Studies by Prasadarao and coworkers indicate that OapA is an additional protein involved in adherence (Prasadarao et al., 1999). OapA is a surface-associated protein responsible for the transparent-colony phenotype in both encapsulated and nonencapsulated *H. influenzae* and is required for efficient colonization in the infant rat nasopharyngeal colonization model (Weiser et al., 1995).

Successful colonization of the respiratory tract by *H. influenzae* requires that bacteria circumvent the mucociliary escalator. Respiratory viral infection, exposure to cigarette smoke and other environmental agents, and inherited ciliary structure abnormalities influence the ability of *H. influenzae* to overcome mucociliary clearance. Based on experiments with human nasopharyngeal tissue in organ culture, *H. influenzae* can disrupt mucociliary function directly, inducing ciliostasis and extrusion of ciliated epithelial cells

(Read et al., 1991; Read et al., 1992; Wilson et al., 1996). Ciliotoxic activity has been attributed to the lipid A moiety of its LOS, to peptidoglycan fragments, and to an immunoglobulin-binding glycerophosphodiesterase called protein D (Janson et al., 1999; Johnson and Inzana, 1986; Kanthakumar et al., 1996). Of note, mutation of protein D results in reduced virulence in a rat model of otitis media, supporting the potential role of this protein in altering ciliary function (Janson et al., 1994).

Persistence of *H. influenzae* on the respiratory mucosa during colonization and in the bloodstream during invasive disease requires that the organism evade the host immune response. The polysaccharide capsule of encapsulated *H. influenzae* protects these bacteria against antibody binding, complement deposition and phagocytosis in nonimmune hosts. A region of the chromosome referred to as "the *cap* locus" controls expression of the capsule in all encapsulated *H. influenzae*. This locus consists of four serotype-specific genes that encode enzymes involved in biosynthesis of the polysaccharide subunit, a set of upstream genes devoted to the export of subunits (*bexA-bexD*), and a group of downstream genes that influence surface assembly of the capsule (Kroll and Booy, 1996). The genetic structure of the *cap* locus facilitates the frequent duplication or loss of this locus via homologous recombination, and the number of *cap* locus copies present in an organism correlates directly with the amount of capsule produced (Roche and Moxon, 1995). Though it may seem disadvantageous for encapsulated *H. influenzae* to readily lose their capsule, it has been shown that loss of capsule results in a marked increase in the ability of bacteria to associate with epithelial cells (St. Geme and Falkow, 1992; St. Geme and Falkow, 1991a). Thus, diminished encapsulation may be an important early step during respiratory tract colonization, a step that is somehow reversed during invasion into the bloodstream.

Another factor presumed to play an important role in immune evasion is the *H. influenzae* IgA<sub>1</sub> protease, which is homologous to the IgA<sub>1</sub> proteases expressed by *N. gonorrhoeae* and *N. meningitidis*. This extracellular endopeptidase cleaves the hinge region in both the serum and secretory forms of IgA<sub>1</sub> and releases the antigen-binding Fab domains from the Fc portion of the molecule, thus eliminating agglutination activity (Plaut, 1978). *Haemophilus influenzae* IgA<sub>1</sub> protease is specific for IgA<sub>1</sub> of humans and higher primates (Qiu et al., 1996) and does not cleave IgA<sub>2</sub> of any species, owing to distinct sequence differences between the hinge regions of IgA<sub>1</sub> and IgA<sub>2</sub>. Two types of *H. influenzae* IgA<sub>1</sub> proteases exist: type 1 proteases cleave the peptide bond between the proline at position 231 and the



serine at position 232, and type 2 proteases cleave the peptide bond between the proline at position 235 and the threonine at position 236 (Kilian et al., 1980; Mulks et al., 1980). Most *H. influenzae* strains produce either one or both types of IgA<sub>1</sub> protease (Insel et al., 1982; Kilian and Thomsen, 1983a; Kilian and Thomsen, 1983b; Male, 1979; Mulks et al., 1982). Many investigators have speculated that the IgA<sub>1</sub> protease is critical for the pathogenesis of *H. influenzae* disease. However, despite the widespread expression of this potential virulence factor in pathogenic *Haemophilus* species and its absence in nonpathogenic species (Senior and Ip, 1999), there is no direct evidence demonstrating its importance in the pathogenic process.

*Haemophilus influenzae* invasion into respiratory epithelium has been proposed as another important mechanism by which the organism evades the host immune response. Microscopic examination of human adenoid tissue and cultured respiratory epithelial cells infected with nontypeable *H. influenzae* suggests that organisms enter respiratory epithelial cells and also pass between cells into deeper tissue via a process called “paracytosis” (Forsgren et al., 1994; van Schilfgaarde et al., 1995). In addition, *H. influenzae* has been reported to reside inside macrophages inhabiting respiratory epithelial tissue (Forsgren et al., 1996). Cellular invasion and paracytosis may provide an explanation for the observation that occasionally the same strain of nontypeable *H. influenzae* persists despite antibiotic treatment. Studies to define the molecular determinants of paracytosis are currently underway (van Schilfgaarde et al., 2000).

Recent studies of *H. influenzae* LOS biosynthesis suggest that the addition of phosphorylcholine (ChoP) may be important to *H. influenzae* persistence in the respiratory tract. LOS production is controlled by three genetic loci, called *lic1*, *lic2*, and *lic3*. Addition of ChoP to LOS is controlled by the *lic1* locus and is phase variable, because of the presence of intragenic CAAT tandem nucleotide repeats in the first gene of this locus, *licA* (Weiser et al., 1997). Surface expression of ChoP decreases bacterial susceptibility to an antimicrobial peptide expressed in the upper respiratory tract

(Lysenko et al., 2000a) and promotes persistence in an infant rat model of nasopharyngeal carriage (Weiser et al., 1998). However, surface expression of ChoP may also be disadvantageous to bacteria because it is the target of C-reactive protein (CRP)-mediated serum bactericidal activity (Lysenko et al., 2000b; Weiser et al., 1998).

The first vaccines against *H. influenzae* type b were developed in the 1970s and consisted of polyribosylribitol phosphate (PRP; the type b polysaccharide) by itself. These plain polysaccharide vaccines were used with success among children over 24 months of age in Finland and parts of the United States and Canada (American Academy of Pediatrics Committee on Infectious Diseases, 1985; Anderson et al., 1972). Unfortunately, these vaccines were poorly immunogenic in children younger than 24 months and had little influence on nasopharyngeal carriage rates (Makela et al., 1977; Peltola et al., 1984; Takala et al., 1991). These problems were addressed in the late 1980s by the development of conjugate vaccines that consisted of PRP bound covalently to an immunogenic carrier protein. Currently, four conjugate vaccines are commercially available, all differing with respect to the size of the polysaccharide, the chemical linkage between the polysaccharide and the carrier protein, and the specific carrier protein employed (see Table 5). As a result of these structural differences, each of the conjugate vaccines has slightly different immunogenic properties, in some cases influencing the recommended immunization schedule. All four vaccine preparations are well tolerated and can be administered at the same physician visit as other vaccines.

In contrast to the situation with *H. influenzae* type b, no vaccine is available for the prevention of disease due to nontypeable *H. influenzae*. Development of such a vaccine has been the subject of intense efforts over the past decade. Among the major challenges along these lines is the identification of an antigen that is highly conserved among diverse strains of nontypeable *Haemophilus* and is also immunogenic. Current candidates include the major outer membrane proteins called P4, P5 and P6, an outer membrane protein called OMP26, the HMW, Hia and

Table 5. *H. influenzae* type b conjugate vaccines licensed for use in children.

Vaccine	Trade name	Polysaccharide	Linkage	Protein carrier	Licensed for Infants <sup>a</sup>
PRP-OMP	PedvaxHIB	Medium	Thioether	<i>N. meningitidis</i> outer membrane protein complex	Yes
HbOC	HibTITER	Small	None	CRM <sub>197</sub> (a nontoxic diphtheria toxin)	Yes
PRP-T	ActHIB/OmniHIB	Large	6-Carbon	Tetanus toxoid	Yes
PRP-D	ProHIBit	Medium	6-Carbon	Diphtheria toxoid	No

<sup>a</sup>Age < 15 months.

Hap adhesins, and Tbp2 (Akkoyunlu et al., 1991; Barenkamp, 1996a; El-Adhami et al., 1999; Green et al., 1994; Kodama et al., 2000; Nelson et al., 1991; Webb and Cripps, 1999; Webb and Cripps, 2000). In addition, in recent studies LOS conjugates and whole cell preparations have been examined for their vaccine potential (Foxwell and Cripps, 2000; Sun et al., 2000).

*H. INFLUENZAE* BIOGROUP AEGYPTIUS *Haemophilus influenzae* biogroup aegyptius represents a distinct subgroup of *H. influenzae* biotype III that is characteristically associated with purulent conjunctivitis. Koch first isolated this organism in 1883 from the eyes of Egyptian children with conjunctival infection (Koch, 1883). Three years later Weeks reported isolation of the same organism from American patients with acute conjunctivitis (Weeks, 1886). As a consequence, *H. influenzae* biotype aegyptius is also known as the Koch-Weeks bacillus. Further studies by Weeks demonstrated that the organism could be transmitted from patients to human volunteers and was responsible for epidemics of conjunctivitis (Weeks, 1887; Weeks, 1895). In the twentieth century, cases of *H. influenzae* biogroup aegyptius conjunctivitis in the United States have been limited to the southern states, particularly during warmer months (Bengston, 1933; Davis and Hines, 1952; Davis and Pittman, 1950). Nevertheless, a 1981 study found that this organism was the most common cause of acute purulent conjunctivitis in children over one month of age (Gigliotti et al., 1981). Although the eye gnat (*Hippelates pusio*) might be an important vector of transmission during epidemics of conjunctivitis (Buehler et al., 1983), *H. influenzae* biogroup aegyptius is also responsible for sporadic conjunctival infection, presumably resulting from direct person-to-person transmission.

In addition to causing acute purulent conjunctivitis, *H. influenzae* biogroup aegyptius causes Brazilian purpuric fever (BPF), a fulminant bacteremic disease of young children first recognized in 1984 in rural Brazil (Brazilian Purpuric Fever Study Group, 1987a; Brazilian Purpuric Fever Study Group, 1987b). Brazilian Purpuric Fever is characterized by fever, abdominal pain, vomiting, rapid progression to purpura, and vascular collapse, with death in approximately 60% of cases. Patients with BPF are typically between the ages of 3 months and 10 years, with the peak incidence occurring between 1 and 4 years. Often the onset of BPF is preceded by an episode of purulent conjunctivitis, suggesting that this illness results from systemic spread of bacteria from an initial site of localized infection.

In 1986, isolates of *H. influenzae* biogroup aegyptius recovered from ten Brazilian children with BPF were subjected to extensive analysis,

including protein profiles, multilocus enzyme electrophoresis, ribosomal RNA gene restriction mapping, plasmid restriction mapping, and seroagglutination reactions. This analysis established that all strains were members of the same clone and distinct from other strains of *H. influenzae* biogroup aegyptius (Brenner et al., 1988). Organisms isolated from two Australian patients with symptoms consistent with BPF were also shown to be *H. influenzae* biogroup aegyptius, but these strains were distinct from the BPF clone identified in Brazil (McIntyre et al., 1987).

Although little is known about the pathogenic mechanisms by which BPF strains cause such serious disease, a number of insights have emerged. First, these strains express both pilus and nonpilus adhesins, which presumably facilitate mucosal colonization (St. Geme et al., 1991b). Second, Porto and colleagues found that BPF strains are relatively serum resistant, which may account in part for their ability to survive in the bloodstream (Porto et al., 1989). Third, BPF strains are cytotoxic for microvascular endothelial cells, a property that possibly underlies the purpura and vascular collapse that occur during BPF (Weyant et al., 1994). Finally, examination of individuals living in areas endemic for BPF shows that young children generally lack serum bactericidal activity against BPF, while most older children and adults have high titers of bactericidal antibodies (Rubin et al., 1993). This observation may provide at least a partial explanation for the age-related incidence of BPF.

### *H. ducreyi*

*Haemophilus ducreyi* is the etiologic agent of chancroid, a sexually transmitted disease characterized by painful genital ulceration and inguinal lymphadenitis. Chancroid is a common cause of genital ulcers in developing countries, but in the United States, it is generally confined to outbreaks in economically disadvantaged minority communities (Schmid et al., 1987). *H. ducreyi* infection is often associated with co-infection by other sexually transmitted pathogens such as *Treponema pallidum* or herpes simplex virus, and chancroid has been shown to be an important cofactor in transmission of HIV (Centers for Disease Control and Prevention, 1998b; Wasserheit, 1992).

Transmission of *H. ducreyi* occurs during sexual contact with an infected person and involves introduction of the organism into a break in the genital epithelium. After an incubation period of four to seven days, a papule develops at the site of infection. The papule then evolves into a pustule over the next two to three days and eventually ruptures spontaneously to form a sharply circumscribed ulcer. Multiple ulcers may appear

and then resolve, with some ulcers occasionally coalescing to form giant ulcers. Roughly 50% of chancroid patients also develop inflamed inguinal lymph nodes, which often become fluctuant and rupture spontaneously. Based on polymerase chain reaction (PCR) studies, asymptomatic carriage of *H. ducreyi* has been reported in female prostitutes but has not been observed in males (Hawkes et al., 1995).

Accurate diagnosis of chancroid relies on cultivation of *H. ducreyi* from the lesion using proper media (synthetic chocolate agar containing 5% fetal bovine serum and 3 µg/ml of vancomycin) and incubation conditions (33°C in a humid, CO<sub>2</sub>-enriched environment). Gram stain of material swabbed from the lesion typically shows characteristic Gram-negative bacteria in a "school of fish" configuration; however, the presence of other bacteria can confound interpretation.

Although the precise mechanism of *H. ducreyi* colonization remains unclear, research over the past decade has identified several potential *H. ducreyi* colonization factors. Virtually all clinical isolates express fine tangled pili with a superstructure distinct from pili of other bacteria (Brentjens et al., 1996). Although experiments to date have been unable to demonstrate that *H. ducreyi* pili possess adhesive activity (Bauer and Spinola, 1999; Frisk and Lagergard, 1998a), it is likely that these organelles promote bacterial interaction with host cells and facilitate colonization. Many strains of *H. ducreyi* also express high molecular weight proteins called LspA1 and LspA2, which are homologous to *B. pertussis* filamentous hemagglutinin (Ward et al., 1998). The absence of these proteins in four strains that are avirulent in a rabbit model of chancroid suggests that they may contribute to colonization. In addition, the *H. ducreyi* GroEL heat shock protein is present on the bacterial surface and appears to promote interbacterial interactions or bacteria-host cell interactions (Frisk et al., 1998b; Parsons et al., 1997).

Tissue damage during genital ulcer formation is likely to involve both bacterial and host factors. One bacterial factor that may play a role in this process is a toxin that was identified based on the ability of *H. ducreyi* to induce distension and delayed cytotoxicity in cultured epithelial cells (Cope et al., 1997). This toxin is referred to as cytolethal distending toxin (CDT) and is composed of three proteins, encoded by genes called *cdtA*, *cdtB* and *cdtC*. Of note, CDT is a member of a family of toxins produced by a variety of Gram-negative pathogens, in all cases inducing similar effects in epithelial cells. CDT cytotoxic activity apparently arises from induction of cell cycle arrest at the G2 phase in target cells (Cortes-Bratti et al., 1999). Experimental evi-

dence suggests that CDT may serve to inhibit the host immune response by inducing apoptosis in T cells (Gelfanova et al., 1999). Interestingly, despite these in vitro observations, comparison of a wild-type strain and an isogenic CDT mutant revealed no difference in virulence in the rabbit experimental infection model (Stevens et al., 1999). Another protein that may influence tissue damage is a cell-associated hemolysin called HhdA, which is capable of lysing cultured human epithelial cells and fibroblasts (Alfa et al., 1996; Palmer and Munson, 1995; Totten et al., 1995; Wood et al., 1999). However, similar to the situation with CDT, a mutant deficient in production of hemolysin and in vitro cytolytic activity remained fully virulent in the rabbit model (Palmer et al., 1998).

In considering the determinants of *H. ducreyi* persistence within genital epithelium, several recent observations provide some insight. In particular, Al-Tawfiq and co-workers found that inactivation of the *hgbA* gene eliminated *H. ducreyi* hemoglobin binding and resulted in reduced pustule formation and bacterial recovery in human volunteers, underscoring the importance of heme acquisition (Al-Tawfiq et al., 2000). In addition, Elkins et al. discovered a variable size outer membrane protein called DsrA, which mediates resistance to killing by normal serum antibody and complement and presumably plays a key role in *H. ducreyi* survival (Elkins et al., 2000).

Efforts to develop a vaccine for the prevention of chancroid have relied on the rabbit infection model to examine the protective effect of immunization with *H. ducreyi* preparations, including whole cell lysates, cell envelopes, purified pili and purified hemolysin (HhdA; Desjardins et al., 1996; Dutro et al., 1999; Hansen et al., 1994). Some degree of success has been reported in each of these vaccination studies, but commercial vaccine production for use in humans will require much additional work.

### Other Human Isolates

The species *H. parainfluenzae*, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. segnis*, *H. aphrophilus* and *H. paraphrophilus* are members of the normal flora in the upper respiratory tract and oral cavity. These organisms can cause local or systemic disease, especially in the setting of a compromised immune system. Besides *H. influenzae*, *H. parainfluenzae* represents the most common pathogen among human *Haemophilus* species and has been reported in association with pharyngitis, otitis media, conjunctivitis, dental abscess, epiglottitis, pneumonia, empyema, septicemia, septic arthritis, osteomyelitis, peritonitis, hepatobiliary infection, meningitis, brain abscess,

epidural abscess, and urinary tract and genital infection. Both *H. aphrophilus* and *H. paraphrophilus* have been associated with a similar spectrum of diseases, along with rare instances of cervical lymphadenitis, lumbar spondylitis, paraspinal abscess and intracranial abscess (Hart et al., 1998; Hung et al., 1997; Margelli and Streit, 1996; Samuel et al., 1997; White et al., 2000). Serious disease in association with *H. aphrophilus* is exceedingly rare in individuals who are immunocompetent and otherwise healthy, but several such cases have been reported, including meningitis complicated by hydrocephalus and humeral shaft osteomyelitis following routine dental work (Adeyemi-Doro et al., 1998; Dewire et al., 1999). *Haemophilus parainfluenzae*, *H. aphrophilus* and *H. paraphrophilus* also have a predilection to cause endocarditis, typically in patients with pre-existing congenital or rheumatic heart disease. Together, these organisms account for approximately 5% of cases of infectious endocarditis and may have been responsible for a portion of "culture-negative" endocarditis reported in the past (Geraci et al., 1977).

A curious association between *H. parainfluenzae* infection and IgA nephropathy has been reported in Japan (Suzuki et al., 1998). Examination of kidney biopsy samples from Japanese children and adults revealed *H. parainfluenzae* antigens in the renal mesangium in patients with IgA nephropathy but not in patients with other renal diseases (Ogura et al., 2000; Suzuki et al., 1994). In addition, sera from patients with IgA nephropathy contained significantly higher levels of IgA reactive with *H. parainfluenzae* antigens than did sera from control patients. Furthermore, tonsillar mononuclear cells from patients with IgA nephropathy were found to be more reactive to *H. parainfluenzae* antigens than were cells from a control group of patients with chronic tonsillitis, producing significantly higher levels of TGF- $\beta$ , IL-10, and *H. parainfluenzae*-specific IgA (Suzuki et al., 2000a; Suzuki et al., 2000b). Finally, intraperitoneal infection of mice with *H. parainfluenzae* resulted in renal deposition of immune complexes containing bacterial antigen and in pathological changes similar to those observed human IgA nephropathy (Yamamoto and Suzuki, 1999). The relevance of these findings to the pathogenesis of IgA nephropathy in Japan and around the world will require further evaluation.

*Haemophilus segnis* is a common resident of dental plaque and gingival scrapings, and the presence of this organism has been correlated with severe periodontal lesions (Petsios et al., 1995). Additionally, *H. segnis* has been associated with rare cases of cholecystitis, appendicitis, pancreatic abscess, and endocarditis (Bangsborg

et al., 1988; Bullock and Devitt, 1981; Carson et al., 1997; Namnyak et al., 1991). Both *H. para-haemolyticus* and *H. paraphrohaemolyticus* have been associated with acute pharyngitis, mouth ulcers, and rare cases of endocarditis. *Haemophilus haemolyticus* has not been associated with any disease.

A recent study examined a collection of *Haemophilus* isolates for IgA1 protease activity. This study detected IgA1 protease production in only one of three *H. para-haemolyticus* strains and in none of the *H. aphrophilus*, *H. paraphrophilus*, *H. segnis* and *H. paraphrohaemolyticus* strains tested (Senior and Ip, 1999). It is interesting to speculate that the relative lack of pathogenicity of the oral *Haemophilus* species may be due in part to the absence of this virulence factor, which is commonly produced by isolates of *H. influenzae*.

### Animal Isolates

*Haemophilus paracuniculus* has been isolated from the gastrointestinal tracts of rabbits with mucoid enteritis (Targowski and Targowski, 1979). However, the pathogenic potential of this organism is unknown, as it appears in the context of a generalized pathologic response of the colon to constipation (Sinkovics, 1976).

*Haemophilus haemoglobinophilus* is part of the normal flora of the preputial sacs of dogs. It has been isolated from dogs with suppurative inflammation of the prepuce (Rivers, 1922), but its identity as the etiologic agent of this particular infection has never been established. Several reports also exist of *H. haemoglobinophilus* associated with human infections. In one case, it was isolated from the middle ear of an agammaglobulinemic child with otitis media (Frazer and Rogers, 1972). In another instance, it was isolated from a boy who had contracted osteomyelitis following a dog bite (Lavine et al., 1974).

*Haemophilus somnus* is an inhabitant of the respiratory and genital tracts of healthy cattle, but like *H. influenzae*, it also can cause disease in its host. In addition to cattle, *H. somnus* has also been isolated from bison and sheep (Ward et al., 1999; Ward et al., 1995). This organism is one of a large group of bacterial and viral agents of bovine respiratory disease (BRD), which encompasses a range of pathologic infectious processes, including bronchiolitis, pneumonia and bronchopneumonia. Additionally, *H. somnus* can cause invasive disease, such as arthritis, bacteremia and meningoencephalitis. The occurrence of *H. somnus* disease is an issue of worldwide importance. The clinical significance of this organism is exemplified by a large-scale Canadian study of feedlot calves, which found that over a three-year period, *H. somnus* infection

accounted for more than 40% of mortality from BRD (Van Donkersgoed et al., 1994a). Furthermore, a study of feedlot calves in Denmark found that *H. somnus* was most often associated with the most severe cases of respiratory disease, such as fibronectin bronchopneumonia (Tegtmeier et al., 1999). Clinical manifestations of *H. somnus* urogenital infections include vesicular adenitis in beef bulls, abortion, and “weak calf syndrome” resulting from perinatal transmission of the organism (Chladek, 1975; Grote-lueschen et al., 1994; Waldhalm et al., 1974). Management of bovine hemophilosis is accomplished with the long-acting antibiotic oxytetracycline, which significantly reduces mortality of BRD but has not been shown to be an effective prophylactic agent (Van Donkersgoed et al., 1994a). Efforts at vaccine development have focused on killed whole organisms, detoxified lipid A, and preparations of unique outer membrane antigens (Groom and Little, 1988; Inzana and Todd, 1992; Silva and Little, 1990; Van Donkersgoed et al., 1995; Van Donkersgoed et al., 1994b). Although many of these agents elicit strong immune responses, none has proven effective enough at preventing disease to warrant use on a large scale.

Several potential virulence factors contributing to *H. somnus* pathogenesis have been described, including a family of high molecular weight peripheral outer membrane Fc (immunoglobulin) receptors that may help this organism evade the bovine immune response in the same manner that protein A contributes to *Staphylococcus aureus* pathogenesis in humans (Corbeil et al., 1997). These immunoglobulin-binding proteins (IgBPs) strongly bind bovine IgM, IgA and IgG2b (Batista et al., 1999; Yarnall et al., 1988). The importance of these proteins in pathogenesis is further supported by their absence in four serum-sensitive isolates from asymptomatic carriers (Cole et al., 1993; Cole et al., 1992). Another property of *H. somnus* that presumably contributes to virulence is the ability to survive within and subvert the defense systems of bovine macrophages. Populations of viable and replicating *H. somnus* have been observed in membrane-bound vacuoles of blood monocytes and alveolar macrophages isolated from experimentally infected cattle. Stimulation of these macrophages with lipopolysaccharide (LPS) or bovine cytokines, such as TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$ , reduced but did not eliminate intracellular bacteria (Gomis et al., 1998). This observation may be explained in part by the ability of *H. somnus* cultures to inhibit nitric oxide production by bovine blood monocytes and alveolar macrophages in vitro (Gomis et al., 1997). Finally, examination of *H. somnus* interaction with bovine polymorphonuclear leukocytes in vitro

suggests that this organism may inhibit neutrophil phagocytosis and respiratory burst and may even induce neutrophil apoptosis (Pfeifer et al., 1992; Yang et al., 1998b).

*H. parasuis* is a member of the normal flora of the respiratory tracts of pigs but also can cause symptomatic respiratory infections and polyserositis (Glässer’s disease). Clinical symptoms of *H. parasuis* infection include neurological signs, anorexia, lameness, swollen joints, difficulty in breathing, fever, wasting, lethargy, cyanosis, and unilateral ear inflammation. More severe manifestations include abortion and even sudden death. In the United States and Canada, *H. parasuis* disease is associated mainly with two antigenic serotypes (more than fifteen serotypes have been documented), with four additional serotypes and nontypeable strains accounting for the majority of remaining cases (MacInnes and Desrosiers, 1999). In addition to LPS, potential virulence factors of *H. parasuis* include a species-specific transferrin-binding protein (Charland et al., 1995) and a neuraminidase (Lichtensteiger and Vimr, 1997).

*Haemophilus paragallinarum* is the etiologic agent of infectious coryza in chickens, a disease of worldwide economic importance. Infection of poultry stocks by this organism has resulted in significant mortality in North and South America, Argentina, India, Morocco, South Africa and Thailand. Symptoms of infectious coryza include nasal discharge, facial swelling, lacrimation, anorexia and diarrhea. Concurrent infection by a variety of other pathogens is associated with more complicated, invasive disease that can include arthritis, septicemia, and spread of *H. paragallinarum* to nonrespiratory sites such as kidney, liver and tarsus (Sandoval et al., 1994). The *H. paragallinarum* isolates from healthy and diseased hosts are typically classified into three antigenic serogroups, and all three serogroups have been implicated in disease. Commercial vaccines against *H. paragallinarum* based on a combination of killed organisms representing each of the three serogroups are widely available around the world. However, local variations in the prevalence of certain serovars within each of the serogroups, and the emergence of new serovar variants in some regions, continue to be an obstacle to international vaccine efficacy.

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# The Genus *Pasteurella*

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## Introduction

Members of the bacterial genus *Pasteurella* usually are regarded as opportunistic, secondary invaders in vertebrates. *Pasteurella multocida* is the only representative of the genus regarded as a major pathogen. Most taxa represent opportunistic invaders that might inhabit the mucosal membranes of the upper respiratory and lower genital tracts of mammals and birds. For most taxa, the pathogenic potential is unknown. The genus *Pasteurella* includes nine named species (*P. multocida*, *P. canis*, *P. stomatis*, *P. dagmatis*, *P. gallinarum*, *P. avium*, *P. volantium*, *P. langaa* and *P. anatis*) and two unnamed taxa (*Pasteurella* species A and species B), all referred to as *Pasteurella* sensu stricto. The type species of the genus *P. multocida* has been separated into three subspecies, *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida* and *P. multocida* subsp. *septica*. In addition, excluding seven species of *Pasteurella* (*[P.] aerogenes*, *[P.] pneumotropica*, *[P.] trehalosi*, *[P.] caballi*, *[P.] mairii*, *[P.] bettyae* and *[P.] testudinis*) from *Pasteurella* has been suggested; however, their names have not been formally changed and the genus name of these species is consequently denoted in brackets. General reference is made to *Pasteurella* sensu stricto.

## Phylogeny

The phylogeny of the genus *Pasteurella* has been investigated by 16S rRNA sequence comparison and rRNA-DNA hybridization (DeLey et al., 1990; Dewhirst et al., 1992; Dewhirst et al., 1993; Olsen et al., 2003). The phylogenetic position of members of *Pasteurella* is shown by analysis of the 16S rRNA sequences of well characterized strains of Pasteurellaceae; however, it remains to be demonstrated that these strains also represent the genetic core of the respective taxa. Thirteen monophyletic groups were recognized in addition to ten taxa not belonging to any group (Fig. 1). Except for the position of “Testudinis” as an out-group, the relative order of these groups and

of the single taxa not included in groups could not be determined. The type species *P. multocida* and the species *P. canis*, *P. stomatis*, *P. dagmatis*, and the unnamed taxon *Pasteurella* species B form the monophyletic core-group of *Pasteurella* sensu stricto (cluster 3B of Dewhirst et al. [1993] and cluster 12 of Olsen et al. [2003]). The two subspecies *multocida* and *gallicida* of *P. multocida* have identical 16S rRNA sequences, and only the sequence of the type strain of *P. multocida* was included in the analysis. The close phylogenetic relationship between the three subspecies of *P. multocida* and *P. canis* has been confirmed by phylogenetic analysis of the *atpD* gene sequence (Petersen et al., 2001a). The avian species, *P. gallinarum*, *P. avium*, *P. volantium* and *Pasteurella* species A, form a separate monophyletic group (cluster 3A of Dewhirst et al., 1993) along with the type strain of *[Haemophilus] paragallinarum* (the brackets indicate that the species is not a true member of genus *Haemophilus*; Mutters et al., 1989). The species *[H.] paragallinarum* is described in The Genus in this Volume *Haemophilus*, but it is phylogenetically related to *Pasteurella* sensu stricto (see also Kilian, 2003). *[Haemophilus] paragallinarum* was not included in the phylogenetic analysis because of its incomplete 16S rRNA sequence.

Also, 16S rRNA sequence comparison showed that *P. langaa* and *P. anatis* were phylogenetically unrelated both to the core group and to the avian group of *Pasteurella*. This is in accordance with previous investigations (Dewhirst et al., 1992; Dewhirst et al., 1993; Olsen et al., 2003). *Pasteurella anatis* was proposed to be included in the new genus *Gallibacterium* (Christensen et al., 2003a), and *P. langaa* was found to be related to *[P.] caballi* by 16S rRNA phylogenetic analysis (Fig. 1). The seven species suggested to be excluded from the genus were scattered on the phylogenetic tree of Pasteurellaceae (Fig. 1). *[Pasteurella] pneumotropica* biotype Jawetz was included in a monophyletic group with other rodent-associated taxa (Dewhirst et al., 1993; Olsen et al., 2003; Fig. 1). *[Pasteurella] aerogenes* was found to be polyphyletic with one group including the type strain being related to *[P.]*



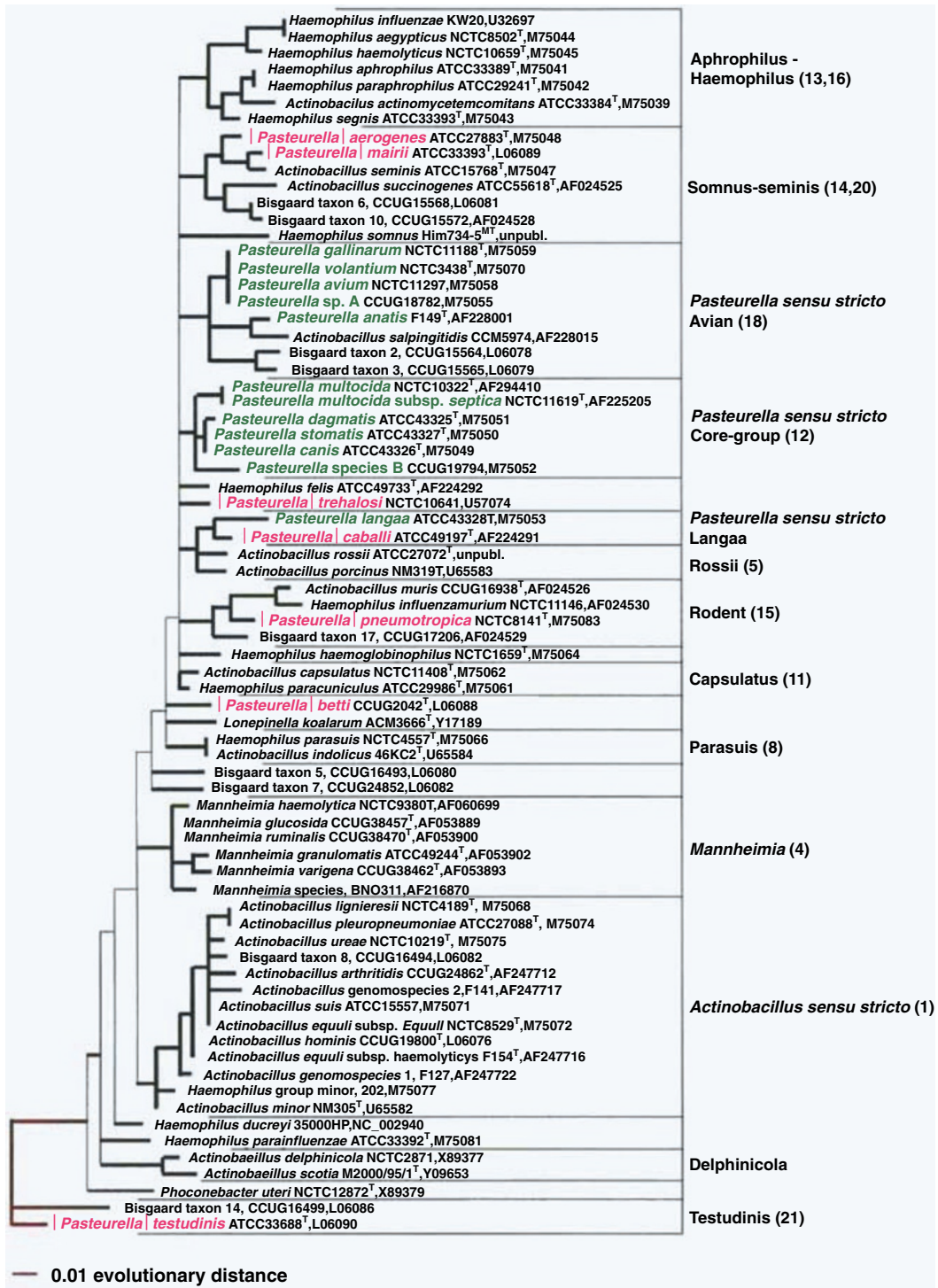


Fig. 1. Maximum likelihood phylogenetic analysis of the bacterial family Pasteurellaceae based on 16S rRNA gene sequence comparison (Olsen et al., 1994) including variable outgroup comparisons and bootstrap analysis. Monophyly for individual groups including support from bootstrap analysis have been marked by bold lines. Members of *Pasteurella* sensu stricto have been typed in green whereas members of the taxa excluded from *Pasteurella* have been typed in red. The naming and numbering of monophyletic groups has been adopted from Olsen et al. (2003).

*mairii* (Fig. 1), while the other was related to [*Actinobacillus*] *rossii* (H. Christensen et al., unpublished observation). Two taxa, [*P.*] *trehalosi* and [*P.*] *betyae* were without close phylogenetic relationship to other taxa of Pasteurellaceae. Additionally, 16S rRNA sequences are available for all four serovars (T3, T4, T10 and T15) of [*P.*] *trehalosi* showing a high similarity of 98.6–99.7%. [*Pasteurella*] *testudinis* finally forms an out-group, together with the unnamed taxon 14 of Bisgaard (Dewhirst et al., 1993; Olsen et al., 2003; Fig. 1). In conclusion, the genus *Pasteurella* is polyphyletic with members included within four out of the thirteen monophyletic groups, just as *Pasteurella* have two out of ten single taxa not belonging to groups of Pasteurellaceae (see Table 1).

## Taxonomy

The taxonomic description includes classification and nomenclature. For the phenotypic characters, refer to Tables 2, 3, 4 and 5.

The current classification and nomenclature of *Pasteurella* has been based mainly on phenotypic characterization and DNA-DNA hybridization. *Pasteurella* is separated from other genera within Pasteurellaceae by the lack of  $\beta$ -hemolysis and the ability of acid formation from (–)-D-fructose, (+)-D-galactose, (+)-D-mannose and sucrose, and lack of acid formation from glycosides and (+)-D-melibiose. A positive test is also obtained with porphyrin and phosphatase (Table 2). According to DNA-DNA hybridization, *Pasteurella sensu stricto* presently contains species interrelated at or above a DNA binding level of 55% (Mutters et al., 1989). Analysis by crossed immunoelectrophoresis and polyamines have confirmed the phylogenetic separation of *Pasteurella sensu stricto* into the core group including the type species, the avian group, in addition to *P. langaa* and *P. anatis* (Schmid et al., 1991; Busse et al., 1997).

### The Core Group of *Pasteurella sensu stricto*

*Pasteurella multocida* has been isolated from both normal and diseased mammals and birds. *Pasteurella multocida* is the type species of the genus and can be separated from other members of *Pasteurella* mainly by positive reactions for ornithine decarboxylase, indole and acid formation from (–)-D-mannitol, and lack of acid production from maltose and dextrin. *Pasteurella multocida* was subdivided into three subspecies, *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida* and *P. multocida* subsp. *septica*, on the basis of differences in acid production from dulcitol and (–)-D-sorbitol (Mutters et al.,

1985a; Table 3). High phenotypic variability has been reported for *P. multocida* including phenotypes for which classification into the existing subspecies categories has not been possible (Biberstein et al., 1991; Fegan et al., 1995; Petersen et al., 1998; see also Identification and Typing). V-factor requiring strains also may occur (Krause et al., 1987). DNA-DNA-binding values of 84–100, 91–100 and 89–100% were found within the three subspecies (*multocida*, *gallicida* and *septica*, respectively), but binding values as low as 55% between the subspecies were also reported (Mutters et al., 1985a). The closest related taxon was *P. dagmatis* with a DNA-DNA-binding value of 62% (Mutters et al., 1985a). A 16S rRNA sequence variation of 1.4–2% was found between *P. multocida* subsp. *septica* and *P. multocida* subsp. *multocida* and subsp. *gallicida*, while subsp. *multocida* and subsp. *gallicida* were nearly identical (Boerlin et al., 2000; Petersen et al., 2001a). Partial *atpD* DNA sequence comparison also showed that the subspecies of *P. multocida* differed, and confirmed 16S rRNA results indicating *P. multocida* subsp. *septica* diverged mostly from the other two subspecies (Petersen et al., 2001a). *Pasteurella canis* has mainly been isolated from dogs. *Pasteurella canis* is urease negative and ornithine decarboxylase positive. Acid is not produced from (+)-L-arabinose, dulcitol, (–)-D-sorbitol or maltose. *Pasteurella canis* is separated from *P. multocida* by the lack of acid production from (–)-D-mannitol. Biotype 1 of *P. canis* exhibits a positive reaction for indole, while biotype 2 strains are indole negative (Mutters et al., 1985a). DNA-DNA relatedness among *P. canis* strains was 80%, while the closest relative of *P. canis* was *P. avium* with a DNA-DNA binding value of 69% (Mutters et al., 1985a). Biovar 2 shares genotypical similarity with *P. multocida*, and *P. canis* should be reclassified only to include the characteristics of biovar 1 (Christensen et al., 2002a). *Pasteurella stomatis* has mainly been obtained from dogs and cats. *Pasteurella stomatis* is indole positive, but urease-negative. No acid is produced from (+)-L-arabinose, (+)-D-xylose, dulcitol, (–)-D-mannitol, (–)-D-sorbitol or maltose. *Pasteurella stomatis* is ornithine decarboxylase negative compared to *P. multocida* and *P. canis*, both being positive. DNA-DNA relatedness among *P. stomatis* strains was 91% and the closest relative to *P. stomatis* was *P. avium* with a DNA-DNA binding value of 81% (Mutters et al., 1985a). *Pasteurella dagmatis* has mainly been isolated from dogs and cats. *Pasteurella dagmatis* is positive for indole and urease and produces acid from (+)-D-maltose, while acid is not produced from (+)-L-arabinose, (+)-D-xylose, dulcitol, (–)-D-mannitol or (–)-D-sorbitol. Small amounts of gas may occur from (+)-D-glucose.

Table 1. Species and species-like taxa of *Pasteurella* with indication of type strain, phylogenetic group, genome size and G+C content.

Species/taxon	Type strain	Phylogenetic group <sup>a</sup>	Genome size ( $\times 10^9$ Da)	G+C (mol%)	References
<i>Pasteurella multocida</i> subsp. <i>multocida</i>	ATCC43137 <sup>T</sup> (= NCTC10322 <sup>T</sup> )	Cluster 12	1.5–1.9	40.8–43.9	Mutters et al., 2003
<i>Pasteurella multocida</i> subsp. <i>septica</i>	ATCC51687 <sup>T</sup> (= NCTC11995 <sup>T</sup> )	Cluster 12	1.5–1.6	41.5–43.5	Mutters et al., 2003
<i>Pasteurella multocida</i> subsp. <i>gallicida</i>	ATCC51689 <sup>T</sup> (= NCTC10204 <sup>T</sup> )	Cluster 12	1.5–1.8	41.2–42.5	Mutters et al., 2003
<i>Pasteurella dagmatis</i>	ATCC43325 <sup>T</sup> (= NCTC11617 <sup>T</sup> )	Cluster 12	1.5–1.7	38.9–41.5	Mutters et al., 2003
<i>Pasteurella canis</i>	ATCC43326 <sup>T</sup> (= NCTC11621 <sup>T</sup> )	Cluster 12	1.4–1.6	37.7–39.8	Mutters et al., 2003
<i>Pasteurella stomatis</i>	ATCC43327 <sup>T</sup> (= NCTC11623 <sup>T</sup> )	Cluster 12	1.5–1.6	40.4–43.5	Mutters et al., 2003
<i>Pasteurella gallinarum</i>	ATCC13361 <sup>T</sup> (= CCUG12391 <sup>T</sup> )	Cluster 18	1.5–1.6	41.2–44.8	Mutters et al., 2003
<i>Pasteurella avium</i>	ATCC29546 <sup>T</sup> (= CCUG12833 <sup>T</sup> )	Cluster 18	1.9	43–45	Mutters et al., 2003
<i>Pasteurella volantium</i>	ATCC14385 <sup>T</sup> (= NCTC3438 <sup>T</sup> )	Cluster 18	1.5–1.9	44–45	Mutters et al., 2003
<i>Pasteurella anatis</i>	ATCC43329 <sup>T</sup> (= NCTC11413 <sup>T</sup> )	Cluster 18	1.8–1.9	39.9–42.3	Mutters et al., 2003
<i>Pasteurella langaa</i>	ATCC43328 <sup>T</sup> (= NCTC11411 <sup>T</sup> )	Cluster 19	1.7–1.9	43.9–45.3	Mutters et al., 2003
Unnamed taxa					
<i>Pasteurella</i> species A	CCUG18782 (= IPDH 280)	Cluster 18	1.7–2.1	44–45.9	Mutters et al., 2003
<i>Pasteurella</i> species B	CCUG19794 (= SSIP683)	Cluster 12	1.9	38.9–40.0	Mutters et al., 2003
<i>Species incertae sedis</i> (named species excluded from <i>Pasteurella sensu stricto</i> )					
[ <i>Pasteurella</i> ] <i>aerogenes</i>	ATCC27883 <sup>T</sup> (= CCUG27904 <sup>T</sup> )	Cluster 14	1.6–2.0	41.8	Mutters et al., 2003
[ <i>Pasteurella</i> ] <i>bettyae</i>	ATCC23273 <sup>T</sup> (= NCTC10535 <sup>T</sup> )	Cluster 9	ND	38.6	Mutters et al., 2003
[ <i>Pasteurella</i> ] <i>caballi</i>	ATCC49197 <sup>T</sup> (= CCUG28833 <sup>T</sup> )	Cluster 9	ND	41–42	Sneath and Stevens, 1990
[ <i>Pasteurella</i> ] <i>mairii</i>	ATCC49633 <sup>T</sup> (= NCTC10699 <sup>T</sup> )	Cluster 14	ND	43.4	Mutters et al., 2003
[ <i>Pasteurella</i> ] <i>pneumotropica</i>	ATCC35149 <sup>T</sup> (= NCTC8141 <sup>T</sup> )	Cluster 15	1.5–1.6	40.3–42.8	Mutters et al., 2003
[ <i>Pasteurella</i> ] <i>trehalosi</i>	ATCC29703 <sup>T</sup> (= NCTC10370 <sup>T</sup> )	Cluster 10	1.8	42.6	Sneath and Stevens, 1990
[ <i>Pasteurella</i> ] <i>testudinis</i>	ATCC33688 <sup>T</sup> (= NCTC12150 <sup>T</sup> )	Cluster 21	1.6	46.8–47.4	Mutters et al., 2003

Abbreviations: <sup>T</sup>, type strain; and ND, not determined.

<sup>a</sup>For information on clusters, refer to Olsen et al. (2003); see Fig. 1 for 16S rRNA accession numbers.

<sup>b</sup>Strains might be obtained through [(American Type Culture Collection (ATCC); [www.atcc.org](http://www.atcc.org)), [(Culture Collection, University of Göteborg, Dept. of Clinical Bacteriology, Göteborg, Sweden (CCUG); [www.ccug.gu.se](http://www.ccug.gu.se))]], and [(National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom (NCTC); [www.phls.co.uk/services/nctc](http://www.phls.co.uk/services/nctc))].

Table 2. Common phenotypic characters of species classified with *Pasteurella sensu stricto*.<sup>a</sup>

Positive characters <sup>b</sup>	Tween 20
Hugh-Leifson medium, glucose fermentation	Tween 80
Porphyrin test	Pigment
Nitrate, reduction	<i>meso</i> -Erythriol
Alanine aminopeptidase	(-)-D-Adonitol
Phosphatase, alkaline	(-)-L-Xylose
(-)-D-Ribose	(+)-D-Fucose
(-)-D-Fructose	(+)-D-Glucose, gas <sup>d</sup>
(+)-D-Galactose	(+)-L-Rhamnose
(+)-D-Glucose, acid	(-)-L-Sorbose
(+)-D-Mannose	Cellobiose
Sucrose	$\beta$ -Glucosidase (NPG)
Negative characters <sup>c</sup>	(+)-D-Melibiose
Gram stain	(+)-D-Melizitose
Motility, 22 and 37°C	(+)-D-Glycogen
$\beta$ -Hemolysis (bovine blood)	Inulin
Citrate, Simmons	Esculin
Mucate, acid	Amygdalin
Malonate, base	Arbutin
H <sub>2</sub> S/TSI	Gentiobiose
KCN, growth	Salicin
Methyl red, 37°C	(+)-D-Turanose
Voges-Proskauer, 37°C	$\beta$ -N-CH <sub>3</sub> -glucosamide
Nitrate, gas	$\alpha$ -Fucosidase (ONPF)
Arginine dehydrolase	$\alpha$ -Galactosidase
Lysine decarboxylase	$\beta$ -Glucuronidase (PGUA)
Phenylalanine deaminase	$\alpha$ -Mannosidase
Gelatinase	$\beta$ -Xylosidase (ONPX)

Abbreviations: TSI, triple sugar iron; NPG, *p*-nitrophenyl- $\beta$ -D-glucopyranoside; ONPF, *o*-nitrophenyl-D-fucopyranoside; PGUA, 4-nitrophenyl-D-glucopyranosiduronic acid; and ONPX, *o*-nitrophenyl- $\beta$ -D-xylopyranoside.

<sup>a</sup>Includes the 16S rRNA cluster 12 (*P. multocida*, *P. dagmatis*, *P. canis*, *P. stomatis*, and *Pasteurella* species B), cluster 18 (*P. gallinarum*, *P. avium*, *P. volantium*, *Pasteurella* species A, and *P. anatis*), and *P. langaa* according to Olsen et al. (2003).

<sup>b</sup>Characters are positive for 90% or more of strains of each species.

<sup>c</sup>Characters are negative for 90% or more of strains within each species analyzed.

<sup>d</sup>Weak positive reactions observed with *P. dagmatis*.

Table 3. Phenotypic characters used for separation of taxa of *Pasteurella*.<sup>a,b</sup>

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.
<i>P. multocida</i>	+	-	+	+	-	-	-	d	d	d	+	-	-	d
<i>P. multocida</i> subsp. <i>multocida</i>	+	-	+	+	-	-	-	d	-	+	+	-	-	d
<i>P. multocida</i> subsp. <i>gallicida</i>	+	-	+	+	-	-	d	+	+	+	+	-	-	-
<i>P. multocida</i> subsp. <i>septica</i>	+	-	+	+	-	-	-	+	-	-	+	-	-	+
<i>P. canis</i>	+	-	+	+	-	-	-	-	-	-	-	-	-	d
<i>P. stomatis</i>	+	-	-	+	-	-	-	-	-	-	-	-	-	+
<i>P. dagmatis</i>	+	-	-	+	+	-	-	-	-	-	-	+	+/(+)	+
<i>Pasteurella</i> sp. B	+	-	+	d	-	+	-	+	+	-	+	+/(+)	+/(+)	+
<i>P. gallinarum</i>	+	-	-	-	-	-	-	d	-	-	-	+	+	+
<i>P. volantium</i>	+	+	d	-	-	-	-	d	-	d	+	+	+	+
<i>P. avium</i>	+	+	-	-	-	-	-	d	-	-	-	-	-	+
<i>Pasteurella</i> sp. A	+	+	-	-	-	d	+/(+)	d	-	-	d	d	d	+
<i>P. anatis</i>	+	-	-	-	-	-	-	+	-	-	+	-	-	+
<i>P. langaa</i>	- <sup>c</sup>	-	-	-	-	-	-	-	-	-	+	-	-	-

Symbols: +, 90% or more of the strains positive within 1–2 days; (+), 90% or more of the strains positive within 3–14 days; -, less than 10% of the strains are positive within 14 days; and d, 11–89% of the strains are positive.

<sup>a</sup>1. Catalase; 2. Symbiotic growth (V-factor); 3. Ornithine decarboxylase; 4. Indole; 5. Urease; 6. Xylitol; 7. (+)-L-Arabinose; 8. (+)-D-Xylose; 9. Dulcitol; 10. (-)-D-Sorbitol; 11. (-)-D-Mannitol; 12. (-)-D-Maltose; 13. Dextrin; and 14. *p*-Nitrophenyl- $\beta$ -D-glucopyranoside (PNPG).

<sup>b</sup>Incubation temperature was 37°C.

<sup>c</sup>Weak positive reaction might occur.

From Mutters et al. (1985a, b, 1989, 2003).

Table 4. Phenotypic characters of limited value for separation of taxa of *Pasteurella*.<sup>a,b</sup>

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.
<i>P. multocida</i>	+	-	d	-	d	-	d	-	-	d	-
<i>P. multocida</i> subsp. <i>multocida</i>	+	-	d	-	d	-	d	-	-	d	-
<i>P. multocida</i> subsp. <i>gallicida</i>	+	-	d	-	-	-	-	-	-	-	-
<i>P. multocida</i> subsp. <i>septica</i>	+	-	d	-	d	-	d	-	-	+	-
<i>P. canis</i>	+	-	-	-	-	-	-	-	-	d	-
<i>P. stomatis</i>	+	-	-	-	-	-	-	-	-	+	-
<i>P. dagmatis</i>	d	-	d	-	d	-	d	d	d	d	d
<i>Pasteurella</i> sp. B	+	-	-	-	-	-	-	-	-	+	-
<i>P. gallinarum</i>	+	-	d	d	d	d	d	d	d	+	d
<i>P. volantium</i>	+	-	-	-	d	-	d	d	+	+	-
<i>P. avium</i>	+	-	-	-	-	-	-	-	-	+	-
<i>Pasteurella</i> sp. A	+	-	d	-	-	-	-	-	d	+	(+)
<i>P. anatis</i>	d	d	(+)	-	-	-	-	d	d	+	d
<i>P. langaa</i>	d	+	-	-	-	-	-	+	+	-	-

Symbols: +, 90% or more of the strains positive within 1–2 days; (+), 90% or more of the strains positive within 3–14 days; -, less than 10% of the strains are positive within 14 days; and d, 11–89% of the strains are positive.

<sup>a</sup>1. Oxidase; 2. McConkey's medium; 3. Glycerol; 4. (+)-D-Arabitol; 5. (-)-D-Arabinose; 6. *m*-Inositol; 7. (-)-L-Fucose; 8. Lactose; 9. *o*-Nitrophenyl- $\beta$ -D-glucopyranoside (ONPG); 10. Trehalose; and 11. Raffinose.

<sup>b</sup>Incubation temperature is 37°C.

From Mutters et al. (1985a, b, 1989, 2002).

Table 5. Colonial and morphological characters of *Pasteurella*.

Species/taxon	Colonial characteristics on blood agar or chocolate agar	Deviation from coccoid and short rod cell morphology
<i>Pasteurella multocida</i>	Colonies smooth, mucoid or rough on bovine or sheep blood or chocolate agar with light grayish color, and diameter 1–2mm Indole-producing strains have a distinct odor	Pleomorphic rods and short filaments can be seen in broth media and older cultures. Many strains produce capsules
<i>P. dagmatis</i>	Smooth and grayish white, diameter 1–1.5mm	ND
<i>P. canis</i>	Small colonies, diameter 0.5–1.5mm	ND
<i>P. stomatis</i>	Small colonies, diameter 0.5–1.5mm	Bipolarly stained ends in the Gram stain
<i>P. gallinarum</i>	Similar to <i>P. multocida</i>	Bipolar staining occurs
<i>P. avium</i> biovar 1	Colonies on chocolate agar usually smooth, convex, slightly yellowish or grayish-white	Coccoid to pleomorphic rods, singly or in short chains
<i>P. volantium</i>	Similar to <i>P. avium</i> biovar 1, but more yellow	Cells coccoid, singly or in short chains
<i>P. anatis</i>	Circular, smooth, grayish-white, diameter 1.5–2mm	ND
<i>P. langaa</i>	Circular, smooth, grayish-white, diameter 1–1.5mm	ND
<i>Pasteurella</i> species B	Colonies are similar to <i>P. canis</i> or <i>P. stomatis</i>	ND
[ <i>P.</i> ] <i>mairii</i>	Colonies on sheep blood agar are round, grayish, semi-transparent, diameter 1–2mm, and have narrow zone of hemolysis	Cells are small bacilli or coccobacilli
[ <i>P.</i> ] <i>bettyae</i>	Colonies on sheep blood agar are round, grayish, semi-transparent, and have diameter 1–2mm	Cells are small <1 $\mu$ m
[ <i>P.</i> ] <i>caballi</i>	Smooth, grayish/yellowish on blood agar, and have diameter 1.0–1.5mm	Cells are rod-shaped 0.8–1.0 $\times$ 1.3–1.9 $\mu$ m, bipolarly stained, singly or in pairs, and occasionally curved filamentous forms
[ <i>P.</i> ] <i>testudinis</i>	Hemolytic zone on sheep blood agar, and diameter 0.5–1.0mm	Cells pleomorphic rods, 0.2 $\times$ 1.5–2.0 $\mu$ m, and capsule-like surface
[ <i>P.</i> ] <i>trehalosi</i>	On sheep blood agar, round, grayish, semi-transparent, diameter 1–2mm, and pronounced zone of hemolysis	ND

Abbreviation: ND, no deviation.

From Sneath and Stevens (1990) and Mutters et al. (2003).

Positive reactions in urease, maltose and dextrin separate the species from *P. multocida*, *P. canis* and *P. stomatis*, all of which are negative in these characters. The DNA-binding value was 84%

within the species, and the species with the highest level of DNA-DNA relatedness was *P. gallinarum* with 78% (Mutters et al., 1985a). *Pasteurella* species B seems to be associated mainly

with cats and dogs. *Pasteurella* species B is ornithine decarboxylase positive, and acid is produced from (+)-D-xylose, dulcitol and maltose. Acid is not produced from (+)-L-arabinose, (-)-D-mannitol, or (-)-D-sorbitol. Urease is not produced. The species with the highest level of DNA-DNA relatedness to *Pasteurella* species B was *P. stomatis* and *P. canis* with 60% (Mutters et al., 1985a).

### The Avian Group of *Pasteurella* sensu stricto

*Pasteurella gallinarum*, *P. volantium*, *P. avium* and *Pasteurella* species A are phylogenetically related (Dewhirst et al., 1993) and referred to as the Avian group of *Pasteurella* (Olsen et al., 2003) because they have been isolated mainly from birds. All taxa are indole negative compared to the “core-group” of *Pasteurella*, which are positive. In addition, the species *P. volantium*, *P. avium* and *Pasteurella* species A are V-factor dependent. The existence of nicotinamide adenine dinucleotide (NAD)-independent strains (Bragg et al., 1997) still remains to be confirmed genetically. *Pasteurella gallinarum* has been isolated from different lesions of fowl. *Pasteurella gallinarum* is urease and ornithine decarboxylase negative and produces acid from maltose and trehalose, whereas acid is not formed from (+)-L-arabinose, dulcitol, (-)-D-mannitol and (-)-D-sorbitol. High genome relatedness was exhibited between *Pasteurella gallinarum* strains (90% DNA-DNA binding) and the closest relative was *P. dagmatis* with 78% DNA-DNA binding (Mutters et al., 1985a). *Pasteurella volantium* has been obtained mainly from domestic fowl. *Pasteurella volantium* produces acid from (-)-D-mannitol, maltose and dextrin and is separated from *P. gallinarum* by the (-)-D-mannitol fermentation and V-factor requirement. The species produces yellowish colonies on chocolate agar. DNA-DNA relatedness among *P. volantium* strains was 81% and the species with the highest level of DNA-DNA relatedness was *P. gallinarum* with 72% (Mutters et al., 1985a). *Pasteurella avium* has been isolated mainly from the wattles of domestic fowl. *Pasteurella avium* is ornithine decarboxylase and indole negative, and acid is not formed from (+)-L-arabinose, dulcitol, (-)-D-mannitol, (-)-D-sorbitol, maltose, dextrin or raffinose. A negative reaction in (-)-D-mannitol, maltose and dextrin separates *P. avium* from *P. volantium*, which are positive. Biotype 1 strains of *P. avium* require V-factor, while biotype 2 strains do not. The DNA-DNA relatedness among *P. avium* strains was 88%, while the species with the highest level of DNA-DNA relatedness was *P. stomatis* with 81% (Mutters et al., 1985a). Biotype 2 strains

isolated from calves (Mutters et al., 1985b) share genotypical similarity with *P. multocida*, and reclassification with this species has been proposed (Christensen et al., 2002a). *Pasteurella* species A has been isolated from the infraorbital sinuses of chickens. The ornithine decarboxylase test is negative, and acid is not formed from (-)-D-sorbitol. *Pasteurella* species A is positive in (+)-L-arabinose compared to the other species of *Pasteurella*, which are negative. DNA-DNA relatedness was 83% among *Pasteurella* species A strains, and the species with the highest level of DNA-DNA relatedness was *P. gallinarum* with 63% (Mutters et al., 1985a).

*Pasteurella anatis* and *P. langaa* differ from the other eleven taxa of *Pasteurella* sensu stricto in many characters (Tables 3 and 4) in accordance with their distinct phylogenetic positions. *Pasteurella anatis* has been isolated from the intestinal and respiratory tract of ducks, but bovine isolates also have been isolated. *Pasteurella anatis* is urease, ornithine decarboxylase and indole negative, and acid is produced from (+)-D-xylose and (-)-D-mannitol. The lowest level of DNA-DNA relatedness among *P. anatis* strains was 94%, and the species with the highest level of DNA-DNA relatedness was *P. multocida* with 51% (Mutters et al., 1985a). Reclassification of *P. anatis* to *Gallibacterium anatis* has subsequently been proposed on the basis of phylogenetic, geno- and phenotypic characteristics (Christensen et al., 2003a). *Pasteurella langaa* has been isolated from the respiratory tracts of chickens. *Pasteurella langaa* is mostly negative for catalase, while ornithine decarboxylase, indole and *p*-nitrophenyl- $\beta$ -D-galactopyranoside (PNPG) reactions are negative. Acid is produced from (-)-D-mannitol. The species with the highest level of DNA-DNA relatedness was *P. volantium* with 57% (Mutters et al., 1985a).

### Taxa Excluded from *Pasteurella* sensu stricto

On the basis of DNA-DNA hybridizations, seven species were excluded from *Pasteurella* sensu stricto: [*P.*] *pneumotropica*, [*P.*] *aerogenes*, [*P.*] *mairii*, [*P.*] *caballi*, [*P.*] *bettyae*, [*P.*] *trehalosi* and [*P.*] *testudinis* (Mutters et al., 1989; Mutters et al., 2003).

[*Pasteurella*] *pneumotropica* has been isolated from rodents. The species is pheno- and genotypically diverse and only the biovar Jawetz, including the type strain, will be described further. Phenotypic characters separating the [*P.*] *pneumotropica*-complex and related taxa have previously been reported by Mutters et al. (1989). Although meeting the general characters of *Pasteurella*, it becomes obvious from the studies of Ryll et al. (1991) and Nicklas et al. (1993)

that the taxonomy of rodent Pasteurellaceae presently is unclear and should be subjected to major changes as indicated by 16S rRNA sequencing (Fig. 1) and on the basis of DNA-DNA hybridization (Ryll et al., 1991). The DNA-DNA relatedness among [*P.*] *pneumotropica* (Jawetz biovar) strains was 46–95%, and the closest relatives of [*P.*] *pneumotropica* (Jawetz biovar) were found to be [*Actinobacillus*] *muris* with 42% and taxon 3 of Bisgaard with 36% (Piechulla et al., 1985; Ryll et al., 1991).

[*Pasteurella*] *aerogenes* (type strain) was originally isolated from pigs. Subsequent investigations, however, seem to indicate a broader host spectrum (Bisgaard, 1993), just as genetic investigations have demonstrated the need of a revised description of this species (see Phylogeny). The species with the highest level of DNA-DNA relatedness to [*P.*] *aerogenes* was [*P.*] *pneumotropica*, but this was only 25% (Mutters et al., 1985a). So far [*P.*] *mairii* has only been isolated from pigs. It is urease positive and indole negative, and it was sensitive to malachite green and 1% Teepol (Sneath and Stevens, 1990). DNA-DNA hybridization between [*P.*] *mairii* and the type strain [*P.*] *aerogenes* showed only 47% relatedness (W. Mannheim, unpublished). [*Pasteurella*] *aerogenes* differs from *Pasteurella* in phosphatase and gas production from glucose, while [*P.*] *mairii* can be separated from *Pasteurella* on the basis of 4-nitrophenyl- $\beta$ -D-glucopyranoside (NPG), 4-nitrophenyl- $\beta$ -D-glucopyranosiduronic acid (PGUA) and *o*-nitrophenyl- $\beta$ -D-xylopyranoside (ONPX) assays (Magne Bisgaard, unpublished results). [*Pasteurella*] *bettyae* isolated from humans differs from *Pasteurella* in catalase, gas production from glucose, and the inability to produce acid from (-)-D-galactose and sucrose. [*Pasteurella*] *bettyae* and [*P.*] *pneumotropica* (its closest relative) share 29% DNA-DNA relatedness (Mutters et al., 1985a).

[*Pasteurella*] *caballi* has so far only been isolated from horses and from infected horse bite wounds and is separated from *Pasteurella* by its aerogenic capacity and negative catalase reaction. The closest phylogenetic relative of [*P.*] *caballi* is *P. langaa* with 53% DNA-DNA binding (Schlater et al., 1989; Fig. 1). [*Pasteurella*] *trehalosi* is associated mainly with a well-defined systemic disease of young adult sheep. [*Pasteurella*] *trehalosi* has been excluded from *Pasteurella* by hemolysis, weak pigment formation, production of acid from glycosides, and lack of acid formation from (+)-D-galactose, in addition to genotypical differences (Mutters et al., 1985a; DeLey et al., 1990; Dewhirst et al., 1993). A strain of [*P.*] *trehalosi* and the type strain of *Mannheimia haemolytica* shared only 18%

DNA-DNA relatedness; however, the DNA-DNA relatedness values between a strain of [*P.*] *trehalosi* and the type strain of [*P.*] *testudinis* was 62% (Mutters et al., 1985a). [*Pasteurella*] *testudinis*, which seems to be associated with desert tortoises, differs from *Pasteurella* in hemolysis and production of acid from (+)-L-rhamnose, (+)-D-melibiose and glycosides, while no acid is produced from (+)-D-mannose (Snipes and Biberstein, 1982; M. Bisgaard, unpublished results). DNA-DNA relatedness values between the type strain and another representative of [*P.*] *testudinis* was 95%. The species with the highest level of DNA-DNA relatedness was [*P.*] *trehalosi* with 62% (Mutters et al., 1986a).

Recently, taxa of *Pasteurella* have been reclassified with other genera: *P. ureae* was reclassified as *Actinobacillus ureae* (Mutters et al., 1986b), the *P. haemolytica*-complex (including *P. granulomatis*) was reclassified with the new genus *Mannheimia* (Angen et al., 1999), and *Pasteurella lymphangitidis* was excluded from the family (Mutters et al., 1985a). A new species, *P. skyensis*, was recently proposed by Birkbeck et al. (2002); however, this species is probably not a member of Pasteurellaceae because it lacks nitrate reduction capability and 16S rRNA similarity with other members of the family. These taxa will not be described further.

### Colonial Characteristics

Surface colonies of members of the genus *Pasteurella* on bovine blood agar or chocolate agar are circular, slightly raised and regular with an entire margin. The surface of the colonies is normally smooth, shiny and nontransparent with a grayish tinge. A diameter of 0.5–2.0 mm is normally observed after 24 hours of aerobic incubation at 37°C. After further incubation, the size of the colonies might reach a diameter of up to 3 mm (Table 5); however, a significant variation in colonial morphology is observed even within the same species.  $\beta$ -Hemolysis on bovine or ovine blood agar is not seen, but a greenish discoloration may occur. Occasionally, yellowish colonies can be seen especially with strains of *P. dagmatis*, *P. canis* and *P. volantium*. Isolates of *P. multocida* from the respiratory tract of ruminants, pigs and rabbits may form large, watery, mucoid colonies, which may collapse after 48 hours of incubation. Rough colonies of *P. multocida* are formed from filamentous noncapsulated cells (Rimler and Rhoades, 1989). Growth in broth usually causes turbidity, but granular growth may occur (Mutters et al., 2003). Colony morphology of the species excluded from *Pasteurella* does not differ significantly from the general description (Table 5).

## Cellular Morphology

Cells of *Pasteurella* appear as coccobacilli or rods, generally 0.3–1.0 µm in diameter and 1.0–2.0 µm in length. Cells occur singly, in pairs, or less frequently in short chains depending on the growth stage. Pleomorphism occurs in older cultures. The bacteria are Gram negative. Bipolar staining can be observed with Giemsa or Wright's stain, especially in tissues. The bacteria are not acid-fast, do not form endospores, and are nonmotile (Table 5).

## Fimbriae

Pili have been observed with *P. multocida*. Pili were only expressed at higher growth temperatures and are probably involved in adherence to host mucosal cells (Rebers et al., 1988; Isaacson and Trigo, 1995).

## Cell Wall

Polyamine analysis has shown that members of the genus *Pasteurella* possess 1,3-diaminopropane, putrescine, cadaverine, spermidine and spermine in the cell wall. The triamine, symnorspermidine, was found only in the core-group of *Pasteurella sensu stricto* (*P. multocida*, *P. canis*, *P. dagmatis*, *P. stomatis* and *Pasteurella* sp. B; Busse et al., 1997). Further characteristics of true members of the genus *Pasteurella* are high amounts of quinones with the chain length of eight (ubiquinone, menaquinone and demethylmenaquinone; Mutters et al., 1993).

## Capsule

Virulent strains of *P. multocida* usually produce capsules, and the capsular antigens are intimately associated with lipopolysaccharide (LPS; Rimler and Rhoades, 1989). The capsule of *P. multocida* is composed of polyanionic polysaccharides resulting in strong hydrophilic properties (Rimler and Rhoades, 1989; Boyce et al., 2000b). It has further been observed that encapsulated strains are more resistant to phagocytosis and to complement and much more virulent in mice compared to nonencapsulated strains (Boyce et al., 2000b). Hyaluronic acid has been found in capsular material of capsular type A of *P. multocida*, and the biosynthesis pathway has been described (DeAngelis, 1999; Boyce et al., 2000b). The capsular material of serogroup B strains contains monosaccharides, arabinose, mannose and galactose, and serogroup B strains have been found to produce hyaluronidase (Rimler and Rhoades, 1989). The chemical composition of the other capsular types is less

known. The capsular material of isolates belonging to capsular group D showed similarities with hyaluronic acid; however, it was not susceptible to hyaluronidase but was susceptible to heparinase III and chondroitinase AC (Boyce et al., 2000b). The capsular material of serogroup F strains was susceptible to chondroitinase AC, but not to hyaluronidase or heparinase III (Boyce et al., 2000b). The antigenic properties of capsular polysaccharides are used for typing of *P. multocida* (see Identification and Typing).

## Outer Membrane Proteins

This group of molecules includes components of importance for virulence, e.g., a 37.5-kDa porin of *P. multocida* affecting bovine neutrophils (Galdiero et al., 1998). Outer membrane proteins (OMPs) also include molecules with specific antigenic properties, such as LPS interacting with the host immune system. Diversity was observed between OMP profiles of [*P.*] *trehalosi* and *M. haemolytica* (Davies and Quire, 1996). A relationship between *P. multocida*, *P. gallinarum*, [*Haemophilus*] *paragallinarum*, *P. volantium* and *P. avium* was inferred through the binding of a polyclonal antibody raised against a putative porin of *P. multocida* (Lubke et al., 1994; Hartmann et al., 1996). Monoclonal antibodies raised against different OMPs of *P. multocida* allowed the detection of strains of capsular type D of *P. multocida* or of the species *P. stomatis*, *P. gallinarum*, *P. betti*, *Pasteurella* sp. B and *P. canis*, but not other Gram-negative bacteria (Marandi and Mittal, 1995; Marandi and Mittal, 1996). A purified OMP of 87 kDa could be identified in all serotypes of *P. multocida* by Western-blot analysis (Ruffolo and Adler, 1996). The expression of OMPs by *P. multocida* was influenced by iron (Choi-Kim et al., 1991; Zhao et al., 1995; Ruffolo et al., 1998), and crossreaction between antibodies from pigs and fowl with iron-regulated OMPs of *P. multocida* isolated from pigs and fowl was further found (Zhao et al., 1995). In [*P.*] *testudinis*, iron-regulated OMPs were also identified (Snipes et al., 1995), and in vivo propagation led to the expression of additional OMPs (Choi et al., 1989).

## Lipid Polysaccharides

Lipopolysaccharides (LPS) of *P. multocida* consist of lipid A, which is responsible for the endotoxic activity, an oligosaccharide core, and the O-antigenic unit consisting of repeating carbohydrate units. The chemical composition of the oligosaccharide and the O-antigenic unit is 2-keto-3-deoxyoctonate, L-glycero-D-mannose, heptose, glucose and glucosamine. In some



cases galactose, rhamnose, D-glycero-D-mannoheptose, glucosamine, glucosamine-6-phosphate, and galactosamine have been found (Rimler et al., 1984; Rimler and Rhoades, 1989; Conrad et al., 1996). The LPS is believed to form the basis of specificity for the somatic typing system (Rimler and Rhoades, 1989). Capsulation did not affect the LPS profile (Rimler, 1990).

### Antigens

Studies of *Pasteurella* antigens (including LPS, OMP and the toxin protein of *P. multocida*) have been reviewed by Confer (1993). On the basis of average linkage cluster analysis of precipitate values obtained by crossed immunoelectrophoresis a close antigenic relationship between species of the genus *Pasteurella* was found (Schmid et al., 1991).

### Fatty Acids

The fatty acids, C<sub>14:0</sub>, C<sub>16:1</sub>, C<sub>16:0</sub>, 3-OH-C<sub>14:0</sub>, C<sub>18:2</sub>, C<sub>18:1</sub> and C<sub>18:0</sub>, were found in human strains of *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, *P. canis*, *P. stomatis*, *P. dagmatis* and *P. caballi* (Schlater et al., 1989; Holst et al., 1992). The fatty acid profiles of the species of the family Pasteurellaceae were found to be indistinguishable (Schlater et al., 1989; Holst et al., 1992; Mutters et al., 1993). Subsequently the growth medium was found to affect the fatty acid profiles (Boot et al., 1999).

### Other Factors

Serogroups A, B, D and E have been found to produce neuraminidase, which is cell-associated and involved in virulence, although the mechanism has not been determined precisely (Rimler and Rhoades, 1989).

### Habitat

Detailed investigations on the habitat of the different species of *Pasteurella* using phenotypic, as well as genotypic, methods for identification remain to be performed. For the same reason, our present knowledge on location and carrier rates remains uncertain. A habitat outside animals also remains to be demonstrated.

On the basis of previous reviews and subsequent updates, the habitat of *P. multocida* seems very varied compared to other Pasteurellaceae (Bisgaard et al., 1994); however, many publications do not allow separation at subspecies level, but since *P. multocida* subsp. *multocida* is most frequently reported, a very broad host

spectrum for this subspecies is anticipated. *Pasteurella multocida* subsp. *gallicida* has most frequently been isolated from birds, and occasionally from pigs (Bowles et al., 2000; Muhairwa et al., 2000). *Pasteurella multocida* subsp. *septica* has been isolated more frequently from cats than dogs (Dziva et al., 2001; Muhairwa et al., 2001b), and infrequently from humans (Boerlin et al., 2000).

*Pasteurella dagmatis*, *P. canis* biovar 1 and *Pasteurella* species B are mainly associated with the oral and nasal mucosa of cats and dogs and have been isolated from human wound infections resulting from bites inflicted by these animals (Bisgaard, 1993; Frederiksen, 1993). [*Pasteurella*] *bettyae* has been isolated from human Bartholin's gland abscesses and a finger infection. In addition, it has been implicated in genital infections, but is only rarely invasive (Frederiksen, 1993). *Pasteurella avium* has been isolated from domesticated and wild birds (biovar 1) and from the lungs of calves suffering from pneumonia (biovar 2). *Pasteurella volantium* also seems to be bird-associated (Bisgaard, 1993), although a single strain has been isolated from human tongue (Kilian, 1976). Strains of *P. gallinarum* are normally associated with different pathological lesions of poultry (including chronic fowl cholera in chickens; Bisgaard, 1993), but isolation from guinea fowls (Mohan et al., 2000) and pigs (Blackall et al., 2000b) has also been reported. Isolates from other hosts probably represent misidentification (Christensen et al., 2002b). *Pasteurella* species A has been reported from both domesticated and wild birds (Bisgaard, 1993). *Pasteurella anatis* has been isolated from the intestinal and respiratory tracts of ducks and geese. Bovine strains seem to be isolated less frequently (Christensen et al., 2003a). *Pasteurella langaa* has been found in the respiratory tracts of apparently healthy chickens. [*Pasteurella*] *aerogenes* and [*P.*] *mairii* seem to be associated with pigs, which may result in disease conditions including abortions and septicemia in piglets. Several cases of pig or boar bite infections have been reported (Lester et al., 1993) just as [*P.*] *aerogenes* has been isolated from a human case of abortion (Thorsen et al., 1994). [*Pasteurella*] *caballi* has been isolated from respiratory and genital tract infections in horses, from wound infections in humans, and from humans who have had contact with horses, including wound infection after horse bites (Schlater et al., 1989; Bisgaard et al., 1991a; Escande et al., 1997). [*Pasteurella*] *testudinis* has been isolated from Californian desert tortoises (*Gopherus agassizii*; Snipes and Biberstein, 1982). [*Pasteurella*] *trehalosi* is an important pathogen for young adult sheep, where it causes a well-defined systemic

disease (Gilmour and Gilmour, 1989). [*Pasteurella*] *trehalosi* is also commonly associated with healthy lambs and ewes (Al-sultan and Aitken, 1985; Gilmour and Gilmour, 1989). In addition, [*P.*] *trehalosi* has been reported from cattle and pigs (Fodor et al., 1999).

## Isolation

### Collection of Sample Material

Sterile cotton swabs are generally recommended for collection of suspect sample material under as sterile conditions as possible. These are streaked onto blood agar plates or other enriched isolation media such as brain heart infusion broth. Blood from cattle with suspected hemorrhagic septicemia that is swabbed from the heart or other affected tissues within a few hours after death can be used for isolation. If the carcass has undergone considerable decomposition, the bone marrow may be examined. In pigs and other animals with atrophic rhinitis, the affected nasoturbinalia are swabbed, and the swabs are streaked onto blood agar plates or incubated further in brain heart infusion broth (Amigot et al., 1998). Chickens suspected of being carriers of *P. multocida* are sampled from the cloaca or upper respiratory mucosal surfaces (Lee et al., 2000).

### Enrichment and Isolation Procedures

For standard isolations, bovine or sheep blood or chocolate agar may be used. By tradition, the V-factor-requiring species are isolated on media supplemented with 5% serum or blood and crossinoculated with a *Staphylococcus* or an *Acinetobacter* strain to provide the V-factor (Mutters et al., 2003). *Pasteurella multocida* can be obtained from contaminated material by subcutaneous or intraperitoneal inoculation of mice with subsequent isolation from the spleen (Carter and De Alwis, 1989; Muhairwa et al., 2001a). Several selective media have been developed for isolation of *P. multocida* (Rimler and Rhoades, 1989; Moore et al., 1994; Lee et al., 2000), just as transport media have been investigated (Kawamoto et al., 1997).

## Identification and Typing

The first step for identification of the *Pasteurella* is to select single colonies suspected to represent members of Pasteurellaceae and to make a broth subculture for replating, thereby ensuring a pure culture. Then the properties of the culture (Gram negative, nonmotile, oxidase and catalase positive, nonhemolytic, phosphatase positive, breaks

down glucose fermentatively without gas formation in Hugh and Leifson's medium, and does not grow in the presence of 4.5% NaCl) are confirmed. Characters stated in Table 2 can be used for separation of *Pasteurella* from other genera of the family Pasteurellaceae in combination with characters used for separation of taxa of *Pasteurella* (Tables 3 and 4). The use of host range (Table 6) should be considered to improve the diagnostic level. Inclusion of reference strains for control of media and tests is highly recommended.

For the separation of *Pasteurella* from other genera of Pasteurellaceae, it is important to make a distinction between the indole positive species (*P. multocida*, *P. canis*, *P. stomatis*, *P. dagmatis* and *Pasteurella* sp. B primarily associated with mammals) and indole negative species (the avian-associated species, *P. gallinarum*, *P. volantium*, *Pasteurella* sp. A, *P. avium*, *P. anatis* and *P. langaa*). Members of *Pasteurella* sensu stricto never exhibit hemolysis on ordinary media. The only *Pasteurella* species exhibiting gas production is *P. dagmatis*. With the exception of *P. dagmatis*, all species of *Pasteurella* are urease-negative, while members of *Actinobacillus* are positive. Acid formation has not been found in (+)-L-rhamnose, and *m*-inositol fermentation is only observed with some *P. gallinarum* isolates. *Pasteurella* produce acid from (–)-D-fructose, (+)-D-mannose and (+)-D-galactose in contrast to many *Haemophilus* and *Actinobacillus* species. Differences in (+)-D-mannose utilization and indole production separate the genera *Mannheimia* and *Pasteurella* (Mutters et al., 2003) (Table 2).

As stated previously, extended characterization and the inclusion of reference strains are highly needed to allow correct identification at the species level (see also Taxonomy). The differential characteristics of species of *Pasteurella* are given in Table 3. Maltose fermentation, in certain cases, may not be a reliable character for identification of *P. multocida* because maltose-positive strains of this species have been reported (Petersen et al., 1998). Identification of *P. gallinarum* from other sources than birds (Bisgaard and Mutters, 1986; Boot and Bisgaard, 1995) and, in rare cases pigs, should be questioned and further identification performed by 16S rRNA sequencing (Christensen et al., 2002b). Full sequencing of 16S rRNA genes and cooperation with reference laboratories are recommended in doubtful cases.

Commercial test systems are of little value in the identification of *Pasteurella* to the species level (Inzana et al., 2000). Polymerase chain reaction (PCR) presently represents the most promising tool for semiautomated identification (see below; Christensen et al., 2003b). PCR tech-

Table 6. Hosts and diseases of *Pasteurella*.

Species/taxon	Mainly isolated from	Main diseases	References
<i>Pasteurella multocida</i>	See below for each subspecies	Fowl cholera, atrophic rhinitis, hemorrhagic septicemia, pneumonia and bite infections	Adlam and Rutter, 1989 Bisgaard, 1993
<i>Pasteurella multocida</i> subsp. <i>multocida</i>	Mammals, including humans and birds		
<i>Pasteurella multocida</i> subsp. <i>septica</i>	Dogs, cats and poultry		
<i>Pasteurella multocida</i> subsp. <i>gallicida</i>	Poultry, pigs, cats, and cattle		
<i>Pasteurella dagmatis</i>	Dogs, cats, and humans	Local infections and septicemia	Bisgaard, 1993
<i>Pasteurella canis</i> biovar 1	Dogs and bites inflicted by dogs	Dog-bite wounds	Bisgaard, 1993
<i>Pasteurella canis</i> biovar 2	Calves	Pneumoniae	Bisgaard, 1993
<i>Pasteurella stomatis</i>	Dogs and cats	Bite infection	Frederiksen, 1993
<i>Pasteurella gallinarum</i>	Fowl	Respiratory- and genital tract infections	Bisgaard, 1993
<i>Pasteurella avium</i> biovar 1	Domesticated and wild birds	Commensals	Bisgaard, 1993
<i>Pasteurella avium</i> biovar 2	Calves	Pneumonia	Bisgaard, 1993
<i>Pasteurella volantium</i>	Domesticated and wild birds	Disease potential unclear	Bisgaard, 1993
<i>Pasteurella anatis</i>	Ducks and geese, and bovine isolates also occur	Disease potential unclear	Bisgaard, 1993 Christensen et al., 2003b
<i>Pasteurella langaa</i>	Chickens	Commensals	Bisgaard, 1993
<i>Pasteurella</i> species A	Chickens, turkeys, and pigeons	Disease potential unclear	Bisgaard, 1993
<i>Pasteurella</i> species B	Cats and dogs and wounds inflicted by these animals	Dog bite wounds and cat scratches	Bisgaard, 1993
[ <i>Pasteurella</i> ] <i>aerogenes</i>	Pigs, rabbits, cattle, and dogs	Abortion in pigs	Bisgaard, 1993
[ <i>Pasteurella</i> ] <i>bettyae</i>	Humans	Bartholin's gland abscesses and finger infections	Mutters et al., 2003
[ <i>Pasteurella</i> ] <i>caballi</i>	Horses and humans	Respiratory and genital tract infections Wound infections	Schlater et al., 1989 Bisgaard et al., 1991a Escande et al., 1997
[ <i>Pasteurella</i> ] <i>mairii</i>	Pigs	Abortion and septicemia	Bisgaard, 1993
[ <i>Pasteurella</i> ] <i>pneumotropica</i>	Rodents	Pneumonia and local infections	Bisgaard, 1993
[ <i>Pasteurella</i> ] <i>testudinis</i>	Tortoises	Respiratory tract disease	Snipes and Biberstein, 1982 Snipes et al., 1995
[ <i>Pasteurella</i> ] <i>trehalosi</i>	Lambs and sheep	Systemic disease	Gilmour and Gilmour, 1989 Fodor et al., 1999

niques should be preferred when isolates are nonpathogenic to mice (Townsend et al., 2000).

### PCR Tests

PCR tests have been developed for specific detection of *P. multocida* (see Christensen et al., 2003b). *Pasteurella multocida* can be detected at the species level by a PCR targeting the *rrl* gene (Mifflin and Blackall, 2001) or by targeting of an unknown gene (Townsend et al., 1998). Species-specific detection may also be used to target the *psl* gene coding for the P6-like protein, but this test needs verification by hybridization (Kasten et al., 1997). Isolates of *P. avium* biovar 2 and *P. canis* biovar 2 also are detected by *P. multocida*-specific PCR (Townsend et al., 1998; Mifflin and Blackall, 2001); however, this is probably related to misclassification because *P. avium* biovar 2, *P.*

*canis* biovar 2, and *P. multocida* share 16S rRNA gene sequences (Dewhirst et al., 1993; Christensen et al., 2002a). A PCR test specific at the biovar level has also been reported for [*P.*] *pneumotropica* (Kodjo et al., 1999b). Bacterial reference and type strains for use as positive controls are listed in Table 1. Positive control strains are of importance for verification of DNA extracted from swabs and to document the performance of the PCR. Negative controls without DNA are included to detect contaminating DNA.

PCR might be performed with DNA extracted directly from swabs or from bacteria isolated on plates. Both strategies allow pre- and post-mortem identification. For fast identification and to limit overgrowth by other bacteria, PCR performed directly on DNA extracted from swabs is preferred. Swabs are soaked in phosphate-

buffered saline, the cell debris spun down and the supernatant centrifuged to collect a pellet containing the bacteria, which is treated with proteinase K (see Chen et al., 1996). For subsequent processing, reference is made to the specific tests.

### In Situ Hybridization

An in situ hybridization test for *P. multocida* based on the recognition of a specific part of the 16S rRNA sequence has been described, and the presence of *P. multocida* verified in sections of infected chicken and pig lungs (Mbutia et al., 2001). The test was recommended for verification of pure cultures of *P. multocida* on smears as well as for histopathological detection of *P. multocida* within infected tissues. Similar to the PCR tests described for *P. multocida*, the in situ hybridization test also detects biovar 2 strains of *P. avium* and *P. canis*, which are routinely misclassified and should be reclassified with *P. multocida* (Christensen et al., 2002a).

### Typing Methods

Typing methods are used for detailed characterization to investigate the diversity of taxa of *Pasteurella* and for the study of pathogenesis and epidemiology. If the discriminatory power of these methods is sufficient, they can be used to collect data about certain members of the bacterial population and their relation to disease and hosts.

### Biotyping

Major differences in phenotypical characters have been reported for *P. multocida* (Tables 3 and 4) and have been utilized for biotyping (Heddleston, 1976; Biberstein et al., 1991; Bisgaard et al., 1991b; Fegan et al., 1995). *Pasteurella canis* and *P. avium* are also separated in two biovars (Mutters et al., 1985b; Mutters et al., 1985a). On the basis of differences in reactions for  $\beta$ -glucosidase and production of acid from glycosides (esculin, amygdalin and gentiobiose), three biotypes have been reported for [*P.*] *trehalosi* (M. Bisgaard, 2002, unpublished observation). Biotyping also has been used within the former [*P.*] *haemolytica* complex in ruminants and avian species, within [*P.*] *aerogenes* and taxon 2 and 3 of Bisgaard (Bisgaard, 1993). Biotyping of *P. multocida* has previously been discussed by Christensen and Bisgaard (Christensen and Bisgaard, 1997; Christensen and Bisgaard, 2000). Biotyping seems most relevant for taxonomic investigations.

### Serotyping

**CAPSULAR SEROTYPING** The capsular typing system reported by Carter (1955) is based upon passive hemagglutination of erythrocytes sensitized by capsule antigen. Five capsule types (A, B, D, E and F) are distinguished (Rimler and Rhoades, 1987; Rimler and Rhoades, 1989). Isolates causing hemorrhagic septicemia belong to serogroups B or E, and isolates causing fowl cholerae to serogroup A. Isolates causing atrophic rhinitis usually belong to serogroup D (Boyce et al., 2000b). So far, capsular typing by the passive hemagglutination test has only been performed in a few laboratories around the world, and presumptive identification of capsular types A, D and F by capsule depolymerizations with mucopolysaccharidases has been reported (Rimler, 1994). Subsequent cloning and sequencing of the entire capsular biosynthetic loci of *P. multocida* strains X-73 (A : 1; Chung et al., 1998) and M 1404 (B : 2; Boyce et al. 2000a) and nucleotide sequence analysis of the biosynthetic region from each of the remaining three capsule types D, E and F identified capsule-specific regions and allowed development of a highly specific multiplex capsular PCR assay (Townsend et al., 2001), which is now used as an alternative to the passive hemagglutination assay. Recently problems with the separation of capsular types A and F in the test have been reported (Townsend et al., 2002). The reference strains for the *P. multocida* capsular types should be requested from national veterinary reference laboratories (e.g., The Royal Veterinary College, London; The National Animal Disease Center, Ames, Iowa, United States) or strain collections like American Type Culture Collection (ATCC), Culture Collection, University of Göteborg, Dept. of Clinical Bacteriology, Göteborg, Sweden (CCUG) and National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom (NCTC).

**SOMATIC SEROTYPING** Sixteen serotypes (1 through 16) were recognized by somatic antigen typing on the basis of the gel diffusion precipitin tests of Heddleston et al. (1972). Somatic serotyping has also been done by tube agglutination (Namioka, 1978), but this method is not widely used, as judged by references in the literature. Serological typing of *P. multocida* has been reviewed and discussed by Rimler and Rhoades (1989). Comparative studies by Brogden and Packer (1979) indicated that a serotype determined by the capsular typing system did not correlate with somatic serotyping. Four serovars, T3, T4, T10 and T15, have been described for [*P.*] *trehalosi* (Biberstein, 1978; Fraser et al., 1982). High homogeneity of LPS profiles was found

within [*P.*] *trehalosi*. Four OMP profiles were found in [*P.*] *trehalosi*, but only one was serotype specific. Serotypes T4 and T15 were suggested to be highly related on the basis of OMP and LPS profiling (Davies and Quirie, 1996).

### Typing of Virulent Members of *Pasteurella*

**HEMORRHAGIC SEPTICEMIA** Specific detection of genotypes with the potential of causing hemorrhagic septicemia belonging to serotype B:2 was possible by a PCR targeting the 16S to 23S rRNA spacer (Brickell et al., 1998), and serotypes B:2, B:5 and B:2,5 with another PCR test (Townsend et al., 1998). In rare cases, serotype D could also be detected (Townsend et al., 2002). Using the PCR protocol for isolates of *P. multocida* causing hemorrhagic septicemia, good correlation to an enzyme-linked immunosorbent assay (ELISA) was found (Brickell et al., 1998). *Pasteurella multocida* strain ATCC 6530 might be used as a positive control with this test.

**FOWL CHOLERA** Tools for typing and identification of virulent strains of *P. multocida* associated with fowl cholera remain to be developed.

**PORCINE ATROPHIC RHINITIS** Serological detection of toxinogenic *P. multocida* is possible using an ELISA (DAKO PMT ELISA) based upon monoclonal antibodies raised against the toxin (Foged et al., 1988). Virulent genotypes of *P. multocida* causing porcine atrophic rhinitis might also be identified by PCR, and in several tests, the gene *toxA* (coding for the toxin) has been used as the target (Nagai et al., 1994; Kamp et al., 1996; Lichtensteiger et al., 1996; Hotzel et al., 1997). (See also Christensen et al. [2003b] for a comparison of these PCR tests.) Good correspondence has been reported between detection by PCR and by in vitro and in vivo detection methods of the toxic protein of *P. multocida* (Nagai et al., 1994; Jong et al., 1996; Kamp et al., 1996; Lichtensteiger et al., 1996). *Pasteurella multocida* strain NCTC 12178 might be used as a positive control for *toxA*-positive isolates.

**LEUKOTOXIN** Green et al. (1999) were able to differentiate between cytotoxic and noncytotoxic [*P.*] *trehalosi* strains using PCR for the coding region of *lktA* (leukotoxin). The PCR method of Fisher et al. (1999) yielded bands of the expected size in selected strains of *P. trehalosi*, *Mannheimia haemolytica*, *M. glucosida*, *M. granulomatis* and *M. varigena* (Fisher et al., 1999; J. Larsen, unpublished observation), which shows that the detection of the *lkt* gene is not allowing species-specific detection of [*P.*] *trehalosi*, since this gene is also found in *Mannheimia* spp. (Fisher et al., 1999; Green et al., 1999).

**MULTILOCUS ENZYME ELECTROPHORESIS** Multi-locus enzyme electrophoresis (MLEE) was used by Blackall et al. (Blackall et al., 1998; Blackall et al., 1999) to investigate the population structure of *P. multocida* and for the epidemiological study of fowl cholera in turkeys.

**DNA-BASED TYPING METHODS** These methods are required for epidemiological investigations because biotyping and serotyping methods do not have enough discriminatory power to trace outbreak-clones of *Pasteurella*. Individual serotypes might include virulent as well as avirulent isolates.

**RESTRICTION ENDONUCLEASE ANALYSIS** Restriction endonuclease analysis (REA) typing with *SmaI-SalI* digestion was used by Snipes et al. (1989) to study the transmission of isolates of *P. multocida* from wild animals to turkeys. REA typing with *BglII* digestion was later used to separate vaccine strains of *P. multocida* from outbreak strains (Kim and Nagaraja, 1990). REA typing based upon *HhaI* digestion was evaluated for separating wildtype vaccine strains and serotypes of *P. multocida*, and it was concluded to be useful for accurate identification and epidemiologic studies (Wilson et al., 1993). REA typing using *HpaII* for digestion of DNA was used for epidemiological studies of porcine pasteurellosis and fowl cholera caused by *P. multocida* (Christensen et al., 1998; Blackall et al., 2000b; Muhairwa et al., 2000). REA typing is useful for fast separation of small numbers of very closely related isolates, and for the same reasons *HpaII* digestion has been used most widely, recently.

**RANDOM AMPLIFIED POLYMORPHIC DNA** Random amplified polymorphic DNA (RAPD) was used for genotypic separation of isolates of *P. multocida* obtained from various animal sources in Zimbabwe. Poor relationship between RAPD-genotype, host and disease was obtained. Porcine isolates tended to cluster together as did cattle isolates from hemorrhagic septicemia (Dziva et al., 2001). Repetitive extragenetic palindromic (REP)-PCR fingerprinting was of great value for epidemiological typing of *P. multocida* to separate isolates from different outbreaks (Gunawardana et al., 2000; Amonsin et al. (2002)) identified certain turkey associated clones by the technique. REP-PCR was used for epidemiological investigation of human isolate of *Pasteurella multocida* and was also found valuable for identification to the subspecies level (Chen et al., 2002). Enterobacterial repetitive intergenic consensus (ERIC)-PCR was not sufficiently discriminatory for epidemiological study of *P. multocida* isolated from dogs (Loubinoux et al., 1999). RAPD represents the method of

choice for fast screening of many isolates among which high genotypic resolution is not obvious.

**RIBOTYPING** Ribotyping based upon *EcoRI* digestion was used by Snipes et al. (1989) for fingerprinting of *P. multocida* isolated from turkeys and wildlife, while ribotyping using *HindIII* digestion later was concluded to be useful for epidemiological studies of toxin-producing *P. multocida* (Fussing et al., 1999). Petersen et al. (1998) used ribotyping for identification of phenotypic variant strains of *P. multocida*. Ribotyping with *HpaII* digestion was used to study porcine pasteurellosis and fowl cholera caused by *P. multocida* (Christensen et al., 1998; Blackall et al., 2000b; Bowles et al., 2000; Muhairwa et al., 2000). *HpaII* digestion showed slightly higher resolution than *HindIII* digestion in the study of Petersen et al. (1998), but almost the same number of ribotypes in the study of Bowles et al. (2000). When highly diverse collections of *P. multocida* were studied with respect to origin and phenotype by ribotyping using *HpaII* digestion of DNA, high diversity was observed with many clusters and without correlation between these clusters and subspecies of *P. multocida* (Blackall et al., 1998; Petersen et al., 2001a). Consequently, ribotyping based upon *HpaII* digestion is too sensitive for identification at the subspecies level of *P. multocida* and is best suited for epidemiological investigation of strains with only minor genotypical differences.

**PULSED FIELD GEL ELECTROPHORESIS** Pulsed field gel electrophoresis (PFGE) also represents a reliable method for tracing closely related isolates of *Pasteurella*. For macrorestriction digestion, *NotI*, *SalI* and *SmaI* have been used (Blackwood et al., 1996; Kodjo et al., 1999a; Boerlin et al., 2000). PFGE was of great value for epidemiological typing of *P. multocida*, separating isolates from different outbreaks (Gunawardana et al., 2000). Characterization of [*P.*] *trehalosi* resulted in 26 PFGE profiles compared to 20 ribotypes and 15 RAPD types (Kodjo et al., 1999a). PFGE was used to investigate the transmission of *P. multocida* from pet animals to babies suffering from meningitis (Blackwood et al., 1996; Boerlin et al., 2000).

The ability of five DNA-based typing methods (REA, ribotyping, PFGE, REP-PCR and MLEE) to type a set of *P. multocida* isolates was compared by Blackall and Mifflin (2000a). The five methods showed various relationships between the outbreak strains, but the between-method agreement was strong.

**AMPLIFIED FRAGMENT LENGTH POLYMORPHISM**  
Amplified Fragment Length Polymorphism

(AFLP) was used for molecular epidemiologic analysis of avian isolates of *Pasteurella multocida* involved in fowl cholera (Amonsin et al., 2002).

In conclusion, identification and typing will be dominated by molecular-based techniques. For the present, PCR-based tests for species identification and PCR-based "serotyping," and REA and PFGE for the study of highly related isolates seem most valuable.

## Preservation

Survival of *Pasteurella* outside their host and in artificial media seems limited. Survival for about a week is possible on enriched media like blood or chocolate agar stored at room temperature, or better at 4°C in a plastic jar or plastic bag to avoid desiccation. Freezing at -80°C is recommended for longer storage. Cultures will remain viable for years when frozen at -80°C in liquid media such as proteose peptone broth containing 20% glucose or glycerol. For long-term survival, either lyophilization or storage in liquid nitrogen is recommended. Both techniques preclude selection of mutants that occur during repeated subculturing. Survival at -80°C for more than two decades has been observed with suspension cultures in calf serum with 7.5% glucose (Lapage and Redway, 1974) in our laboratory.

## Physiology

Members of *Pasteurella* are aerobic to microaerophilic or are facultatively anaerobic. Increased carbon dioxide may improve the growth of surface cultures of certain isolates. They are chemoorganotrophic with both oxidative and fermentative types of metabolism. The major route for carbohydrate utilization is the pentose phosphate pathway, whereas the citric acid cycle only operates supplementarily (Rimler and Rhoades, 1989). The electron transport system is cytochrome-based with oxygen, nitrate or fumarate as the terminal electron receptor. Nitrate reductase is produced. Most species are V-factor (NAD requirement for growth) and X-factor (requirement for hemin or other porphyrins) independent, but V-factor-requiring strains do occur. So far, V-factor requirement has been reported for *P. avium*, *P. volantium* and *Pasteurella* sp. A (Mutters et al., 1985b) and some isolates of *P. multocida* subsp. *multocida* (Krause et al., 1987). Cysteine, nicotinamide, pantothenate and thiamine are required for growth of *P. multocida* (Rimler and Rhoades, 1989). Growth can be observed in a wide mesophilic range (25–42°C). *Pasteurella multocida*, *P. stomatis*, *P. dagmatis* and *P. gallinarum* have optimal growth

optimum at 36°C. With *P. multocida*, the optimal growth temperature for avian strains might be as high as 42°C, compared to 36°C for mammalian strains.

## Genetics

### Genome Structure

The mol% G+C of the DNA of *Pasteurella* is 37.7–45.9. Most species excluded from *Pasteurella* sensu stricto all fall within this range also, but [*P.*] *testudinis* has a slightly higher value with a G+C content of around 47 mol% (Table 1). The genome molecular weights are 1.4–2.1 × 10<sup>9</sup> Da (Table 1).

The genomic sequence has been determined for an avian strain of *P. multocida* (Pm70) isolated from a fowl cholera-infected chicken and found to be 2,257,487 bp long (1.5 × 10<sup>9</sup> Da), with 2,014 predicted coding regions (May et al., 2001). The variation between DNA sequences of 16S rRNA and *atpD* and *infB* genes of the type strain and strain Pm 70 of *P. multocida* is 1–2% (H. Christensen, unpublished observation). A similar level of genetic divergence has been observed between the subspecies of *P. multocida* (Petersen et al., 2001a) and shows that the genome-sequenced strain Pm 70 is genetically divergent from the type strain of *P. multocida*. Similar but not identical genomic structure of strains of *P. multocida* was found by I-*CeuI* restriction analysis; however, a few bovine strains diverged (Liu et al., 1999). Strains of *P. multocida* have been found to possess six ribosomal operons (Liu et al., 1999; May et al., 2001). Few coding genes have been characterized in *Pasteurella* and exclusively in *P. multocida*. The putative genes for catabolic pathways of asparagine, histidine, leucine, lysine, and phenylalanine were absent in the *P. multocida* genome (May et al., 2001).

Gene expression of *P. multocida* was studied by microarray analysis in relation to nutrient limitation (Paustian et al., 2002). About one third of the presumed genes of the organism changed expression level when nutrient regimes were changed. In rich media, genes involved in energy metabolism, protein-, nucleotide- and lipid synthesis, iron transport and cellular binding were upregulated, whereas genes encoding for amino acid biosynthesis, protein transport, OMP and heat shock proteins were upregulated in nutrient-deficient media (Paustian et al., 2002).

### Description of Virulence Genes

The formation and activity of the dermonecrotic toxin found in some representatives of capsular types A and D of *P. multocida* associated with

atrophic rhinitis in pigs has been described (Hunt et al., 2000). The toxin is acting by inducing increased levels of DNA synthesis in the host cells by stimulating intracellular signalling cascades (Rozenfurt et al., 1990; Lax and Grigoriadis, 2001). The C terminus was found to possess catalytic activity and the N terminus to facilitate cell binding (Busch et al., 2001; Pullinger et al., 2001). The protein toxin is coded by *toxA* as shown by cloning and characterization (Lax and Chanter, 1990; Petersen and Foged, 1990b).

Genes of the repeat-in-toxin (RTX) family have only been found in [*P.*] *aerogenes* and [*P.*] *mairii*, but not in all strains of these species and not in the type strains of these species (Kuhnert et al., 1997). The leukotoxin and its structural gene, *lktA*, has been found in all serotypes of [*P.*] *trehalosi* (Shewen and Wilkie, 1982; Burrows et al., 1993; Davies et al., 2001) and the sequence of *lktCABD* characterized in detail (Davies et al., 2001; Davies et al., 2002). Similar to other members of the RTX gene family, *lktA* encodes the protoxin, *lktC* is responsible for posttranscriptional activation, and *lktB* and *lktD* for transport of the toxin. Leukotoxin has been shown to play a major role in pathogenesis (Petras et al., 1995; Highlander et al., 2000) and was responsible for the β-hemolytic phenotype on blood agar (Murphy et al., 1995; Fedorova and Highlander, 1997). A correlation between PCR-positive isolates for the *lkt*-gene and β-hemolytic activity was found by Fisher et al. (1999), and isolates with β-hemolytic activity had a greater potential to cause disease (Jaworski et al., 1998). Lateral DNA transfer and recombination have been important evolutionary mechanisms in diversification of the *lkt* gene (Davies et al., 2001; Davies et al., 2002).

Except for the *toxA* gene, no genes directly involved in virulence have been found in *P. multocida* by the newest molecular screening techniques, and other virulence factors of *P. multocida* are incompletely understood. In vivo expression technology (IVET) showed in vivo expression in mice of lipoproteins, enzymes involved in pyrimidine synthesis, general biosynthetic and metabolic functions, in addition to proteins with unknown functions (Hunt et al., 2001). Microarray analysis of *P. multocida* showed two gene regions homologous to the filamentous hemagglutinin gene of *Bordetella pertussis*, coding for proteins important in host cell binding and immunity (May et al., 2001). By signature-tagged mutagenesis in a mouse model, genes assumed to be directly involved in virulence were identified homologous to those involved in hemolysin synthesis, hemagglutinin synthesis, *Ton*-dependent transport of iron,

adherence, and capsular synthesis, in addition to genes involved in amino acid and nucleotide biosynthesis (Fuller et al., 2000).

Common to these investigations of gene expression are the activation of iron uptake systems. Iron uptake systems are indirectly related to virulence, since the iron concentration is limited in the host and iron is essential for the function of metabolic electron transport chains of the pathogen. Microarray analysis of *P. multocida* under iron-restricted conditions showed increased expression of the gene homologs *yfe-ABCD*, *fbpABC*, *fecBCD*, *tonB* and *exbBD* involved in iron uptake (Paustian et al., 2001) and further studies with different iron source suggested that different pathway might respond to inorganic and organic source of iron in *P. multocida*. A 95 kDa-OMP protein was extracted from *P. multocida* under iron-depleted conditions, which showed identity to the heme acquisition system receptor for *P. multocida*. Cloning and sequencing of the gene had 98% similarity with the *HasR* homolog of *P. multocida* Pm 70 and a predicted molecular weight of 96 kDa (Prado et al., 2002).

### Fimbria and Capsule

Capsulation and piliation are potential virulence factors and their genes have been characterized (Boyce et al., 2000b; Doughty et al., 2000). The biosynthetic locus of serogroups A and B have been characterized in detail. Both consist of three parts, two involved in capsule translocation and phospholipid substitution and one in formation of activated sugar monomers and assembly of the polysaccharide polymers. The biosynthetic locus of serogroup A consists of 11 open reading frames (ORFs), nine for which functions have been proposed: four genes (*hexABCD*) are involved in export of polysaccharides, and the next five genes (*hyaABCDE*) encode proteins involved in the formation of activated sugar monomers and the assembly of hyaluronic acid. Finally two genes (*phyAB*) are involved in phospholipid substitution of hyaluronic acid before translocation. Fifteen ORFs have been proposed for the capsule biosynthetic locus of serogroup B, seven for which functions have been proposed: the first four (*cexABCB*) are probably involved in export of the polysaccharide, the fifth and the last genes (*lipA* and *lipB*) are suggested to be involved in phospholipid substitution, and nine genes (*hcbABCDEFGHI*) are proposed to be involved in formation and assembly of activated sugar monomers (Boyce et al., 2000a; Boyce et al., 2000b).

The genes of type 4 fimbriae have been characterized in serovars of *P. multocida* (Ruffolo et al., 1997; Doughty et al., 2000).

### Plasmids and Transposons

Plasmids of sizes between 1.3 and 100 kbp have been found in *P. multocida* (Hunt et al., 2000), whereas plasmids between 1.8 and 28 kbp have been observed in [*P.*] *aerogenes* (Kehrenberg and Schwarz, 2001a). Plasmids have been found related to resistance to streptomycin, sulfonamides, tetracycline, penicillins, kanamycin and chloramphenicol (Rimler and Rhoades, 1989; Hunt et al., 2000). The distribution of identical nonconjugative R plasmids of *P. multocida* did not follow the clonal population structure (Ikeda and Hirsh, 1990). The transposon *Tn5706* is the only transposable element identified in *P. multocida* (Hunt et al., 2000).

### Antibiotic Resistance

Genes encoding for resistance to antibiotics have been associated with small plasmids, or with conjugative or nonconjugative transposons (Kehrenberg et al., 2001b). Most of the plasmids associated with resistance are less than 10 kbp (Kehrenberg et al., 2001b). Most antibiotic resistance genes are thought to have been transferred to *Pasteurella* from Enterobacteriaceae or other bacteria, but *tet(H)* has only been found in *Pasteurella* (Kehrenberg et al., 2001b).

*Pasteurella multocida* has been found to carry *tet(H)* and *tet(M)* genes with both plasmid and chromosomal locations (Hansen et al., 1993; Hansen et al., 1996; Chaslus-Dancla et al., 1995; Kehrenberg and Schwarz, 2001a). The tetracycline-resistance genes *tetR* and *tet(H)* were found located in the plasmid-borne *Tn5706* (Kehrenberg et al., 1998). [*Pasteurella*] *aerogenes* carried *tet(B)* or *tet(H)* genes encoding resistance to tetracycline. The *tet(B)* gene was located on *Tn10* and chromosomally integrated. However, a plasmid-location was also reported (Kehrenberg and Schwarz, 2001a; Kehrenberg et al., 2001b).

Genes encoding  $\beta$ -lactams were located on small plasmids of 4.1–4.4 kb in *P. multocida*, while genes encoding aminoglycoside resistance were mainly associated with small plasmids of less than 10 kb in *P. multocida* (Kehrenberg et al., 2001b). A human strain produced ROB-1  $\beta$ -lactamase encoded from a plasmid location (Rosenau et al., 1991). Chloramphenicol resistance was encoded by the resistance genes *catAI* and *catAIII* detected on plasmids of *P. multocida* and [*P.*] *aerogenes* (Vassort-Bruneau et al., 1996; Kehrenberg et al., 2001b).

### Population Genetics

The population structure of *P. multocida* and [*P.*] *trehalosi* has been concluded to be clonal as



determined by MLEE, with genetic diversity of between 0.289 and 0.474 (Davies et al., 1997; Blackall et al., 1998; May et al., 2001). Genotypical characterization of diverse collections of isolates of *P. multocida* and *P. gallinarum* with respect to hosts, diseases and geographic origin has indirectly confirmed the clonal population structure (Dziva et al., 2001; Petersen et al., 2001a; Muhairwa et al., 2001b; Christensen et al., 2002b).

Antibiotic resistance has probably evolved by horizontal spread of genes both within *Pasteurella* and between *Pasteurella* and other bacterial groups (Kehrenberg and Schwartz, 2000). A plasmid with resistance to tetracycline could be found in both *P. multocida* and [*P.*] *aerogenes* (Kehrenberg and Schwartz, 2000).

## Ecology

The natural reservoir of *Pasteurella* seems to be the mucosal membranes of the respiratory, genital and intestinal tract of mammals and birds (Table 6). Most members of *Pasteurella* are regarded as opportunistic pathogens that may colonize and form part of the indigenous flora of the mucous membranes of the respiratory, genital and intestinal tracts of vertebrates; however, certain virulent strains of *P. multocida* might occur as obligate parasites. The fact that *Ascaridia galli* infections (followed by secondary *P. multocida* infection) resulted in more birds with pathological lesions and continued *P. multocida* excretion (Dahl et al., 2002) suggests interactions between *Pasteurella* and members of the indigenous flora of the different mucosal membranes remain to be elucidated (in addition to interactions between *Pasteurella* and the host).

The strongly hydrophilic capsule of *P. multocida* probably aids in the protection of the bacteria against dehydration, improving transmission from host to host and survival in the environment (Rimler and Rhoades, 1989; Boyce et al., 2000b). It has further been observed that encapsulated strains are more resistant to phagocytosis and to complement and are much more virulent in mice compared to nonencapsulated strains (Boyce et al., 2000b).

*Pasteurella multocida* has been isolated worldwide among terrestrial, as well as aquatic, species of mammals and birds. Investigations on the carrier rate of *P. multocida* in healthy commercial poultry flocks and fowl cholera-affected flocks showed that the latter carried *P. multocida* at a significantly higher prevalence in the mucosa of the cloaca than in the pharynx, while the opposite was observed in birds from healthy flocks. A different clone was found in each of four outbreaks of fowl cholera on a farm during a

two-year surveillance period, indicating that extra-animal survival seems limited (Muhairwa et al., 2000). *Pasteurella canis* biovar 1, *P. stomatitis*, *P. dagmatis* and *Pasteurella* sp. B are mainly associated with the oral and nasal mucosa of dogs and cats (Bisgaard, 1993; Ganiere et al., 1993; Muhairwa et al., 2001a). *Pasteurella gallinarum* is normally reported in association with different pathological lesions in poultry (Bisgaard, 1993), rarely in pigs, and isolations from rodents and humans probably represent misidentification (Christensen et al., 2002b). A single isolate has been reported from a normal duck (Muhairwa et al., 2001a). *Pasteurella avium*, *P. volantium*, *Pasteurella* sp. A, *P. langaa* and *P. anatis* are all associated with birds. [*Pasteurella*] *trehalosi* seems to be associated with the mucosal surfaces of the upper respiratory tract of ruminants (Gilmour and Gilmour, 1989). A range of characteristics can be correlated with the parasitic lifestyle of these organisms including requirements for growth factors, low oxygen tolerance, mechanisms for iron competition, surface structures to endure the host immune system, and tendencies for host association between certain taxa and orders of vertebrates, mostly domesticated. *Pasteurella multocida* represents an exception associated with a wide range of hosts; however, the possibility that certain clones are associated with diseases of a particular animal species remains to be investigated.

## Epidemiology

The distribution of members of *Pasteurella* in clinically diseased-, subclinically affected-, and healthy animal populations has been investigated during the last decade by molecular comparisons at the DNA level. The use of molecular methods has tremendously improved tracing of bacterial isolates, especially for taxa like *P. multocida*, which have a broad host reservoir resulting in many potential possibilities for transmission.

Studies based on genotypical analysis have shown a global distribution of taxa investigated (e.g., Fusing et al., 1999; Christensen et al., 2002b; F. Dziwa et al., manuscript submitted). The impact of animal transportation and trade on the distribution pattern is, however, difficult to evaluate.

### Spread of Members of *Pasteurella* Between Animals

In an early molecular study by ribotyping of fowl cholera in turkeys, the outbreak was found to be caused by multiple ribotypes (Snipes et al., 1989). This result seems to be atypical since later

studies have shown that outbreaks most often have been clonal. Blackall et al. (1999) conducted REA, ribotyping and MLEE studies of fowl cholera outbreaks in turkeys and concluded outbreaks were often caused by only a single clone, and different clones were involved in different outbreaks. This conclusion was confirmed by Muhairwa et al. (2000), who found only single clones of *P. multocida* were involved in duck cholera outbreaks as shown by REA and ribotyping; however, some clones were found in more flocks. In respect to porcine isolates, the situation was almost similar when isolates collected from different farms were investigated. Few ribotypes could be isolated from different farms with mostly only a single type isolated on a farm (Bowles et al., 2000). This distribution was explained by movement of pigs between farms. In a more detailed study of porcine pasteurellosis caused by *P. multocida* involving only four outbreaks, a single clone was found responsible for two outbreaks, while two or three types were associated with the other two outbreaks. The presence of two or more types on farms was proposed to be related to the transfer of animals between farms (Blackall et al., 2000b); however, the significance of management remains to be investigated further. Healthy dogs most frequently were colonized by a single REA-type of *P. multocida*, and REA types were probably exchanged between dogs by contact (Loubinoux et al., 1999). The same was found for [*P.*] *trehalosi* where outbreaks were found to be caused by specific clones, as documented by REA-typing and ribotyping. Furthermore, the transmission from ewes to lambs of specific strains was shown (Jaworski et al., 1998).

Transmission of *P. multocida* between wild animals and turkeys was studied systematically by Snipes et al. (1988). *Pasteurella multocida* was isolated from a range of wild animals, and inoculation of turkeys with these isolates also showed differences in virulence. A tendency was demonstrated towards a higher virulence of isolates from wild animals close to turkey farms (Snipes et al., 1988). In a further study it was confirmed that some of the isolates from wildlife shared REA-types with outbreak strains from turkeys (Snipes et al., 1989). The observation that 80% of the outbreaks in poultry in Denmark included flocks that had been in contact with the avifauna, and the fact that a highly virulent clone of *P. multocida* subsp. *multocida* involved in two outbreaks of fowl cholera in wild birds in Denmark in 1996 was subsequently re-isolated from a domestic duck cholera outbreak, confirm that the avifauna represent a risk for industrial production (Christensen et al., 1998; Christensen et al., 1999). The importance of wild birds as a reservoir for fowl cholera was subsequently underlined by experimental studies (Petersen et al., 2001b).

In an extensive study in Tanzania, *P. multocida* was isolated from 0.7% of chickens, 7% of ducks, 4% of dogs and 51% of cats investigated. Although the poultry was scavenging and in contact with dogs and cats, it was not possible to isolate bacteria with identical genotypes (as determined by REA and ribotyping) from poultry, dogs and cats (Muhairwa et al., 2001a, b). It was concluded that most of the colonization of animals, including colonization resulting in disease, was by a single isolate (DNA type); however, the potential for spread of *P. multocida* between different animal groups including the spread of virulent strains, requires further study.

### Spread of Members of Pasteurella Between Animals and Man

There are indications that *P. multocida* is more frequent in chronic respiratory tract disease of farmers than in that of other groups, probably reflecting cross-infection from domestic animals (Frederiksen, 1989). Similar observations have been made in relation to the isolation of [*P.*] *aerogenes* from a human abortus and pig bite wounds (Lester et al., 1993; Thorsen et al., 1994).

Isolates of *P. multocida* associated with meningitis in babies have been traced to pet animals by PFGE. In one case, a human isolate of *P. multocida* subsp. *septica* shared a PFGE profile with an isolate obtained from the house cat (Boerlin et al., 2000). In another case, identity was not found, but *P. multocida* could be isolated both from pets and family members (Blackwood et al., 1996). These studies have documented that isolates of *P. multocida* might be transferred from domestic animals, including healthy pets, resulting in serious diseases in man.

## Diseases and Treatment

*Pasteurella multocida* causes a wide range of diseases in animals. Capsule type A, serotypes 1, 3 and 4, are recognized as the primary cause of fowl cholera in poultry and wild birds (Rhodes and Rimler, 1989; Christensen and Bisgaard, 1997; Table 6). Disease may appear as an acute septicemia characterized by disseminated intravascular coagulation, petechial or ecchymotic hemorrhages, multifocal necroses and fibrinous pneumonia. Chronic infections may involve a variety of types and local infections (Christensen and Bisgaard, 1997; Christensen and Bisgaard, 2000).

*Pasteurella multocida* serotypes B : 2 and E : 2 are associated with hemorrhagic septicemia of cattle, water buffaloes, and occasionally other species, resulting in major economic losses, mainly in Southeast Asia (Carter and Alwis, 1989; de Alwis, 1995). Respiratory diseases in

cattle, including bronchopneumonia in feedlot cattle and enzootic pneumonia of calves less than 6 months old, are mainly associated with capsule type A (Frank, 1989). Outbreaks of septicemia in fallow deer have also been reported (Eriksen et al., 1999). Infections of major economic importance in pigs include atrophic rhinitis and bronchopneumonia (Chanter and Rutter, 1989; Gardner et al., 1994; Nagai et al., 1994). These syndromes are caused by capsular types A and D. Members of *P. multocida* belonging to capsule types A and D may also cause pneumonia in pigs (Chanter and Rutter, 1989). Severe cases of atrophic rhinitis are mainly associated capsule type D. Pulmonary lesions may result in blood-borne dissemination to the kidneys in pigs (Buttenschøn and Rosendal, 1990). Recently, *P. multocida* was proposed to be associated with porcine dermatitis and nephropathy syndrome (PDNS; Lainson et al., 2002).

Atrophic rhinitis caused by *P. multocida* has also been reported in goats and rabbits (Baalsrud, 1987; DiGiacomo et al., 1989). In addition to major diseases in production animals, *P. multocida* is recovered from a wide range of sporadic infections in many other species, including laboratory animals (Manning et al., 1989), dogs and cats (Mohan et al., 1997) and other mammals (DiGiacomo et al., 1989).

*Pasteurella multocida* and other species of the 16S rRNA cluster 12 are considered as zoonotic pathogens (Bisgaard et al., 1994). Most human infections associated with *Pasteurella* species result from animal bites. The species usually observed in these infections are *P. multocida* subsp. *multocida* and subsp. *septica*, *P. canis*, *P. dagmatis* and *P. stomatis* (Holst et al., 1992; Escande and Lion, 1993; Matsui et al., 1996). Other infections in humans associated with *P. multocida* include necrotizing fasciitis (Hama-moto et al., 1995), chronic lung abscess (Machiels et al., 1995), endocarditis (Genne et al., 1996), meningitis (Boocook and Bowley, 1995; Armstrong et al., 2000), pulmonary diseases (Ory et al., 1998), peritonitis (Wallet et al., 2000), septicemia (Greif et al., 1986), periocular abscess and cellulitis (Hutcheson and Magbalon, 1999), and granulomatous hepatitis (Chateil et al., 1998).

*Pasteurella gallinarum* was originally associated with enzootic, chronic fowl cholera in chickens (Hall et al., 1955). In addition, *P. gallinarum* has been reported from acute fowl cholera (Yadav et al., 1977). Other lesions include conjunctivitis, sinusitis, tracheitis, airsacculitis, hepatitis, abscess formation, endocarditis, salpingitis, oophoritis, peritonitis and synovitis (Christensen et al., 2002b). The reasons for differences in virulence between strains of *P. gallinarum* remain to be investigated (Shivaprasad and Droual, 2002).

[*Pasteurella*] *trehalosi* is mainly associated with sheep in which they may cause acute sys-

temic pasteurellosis. The pathogenesis remains unclear since pneumonia in specific-pathogen-free (SPF) lambs has not been reported with [*P.*] *trehalosi*. In addition, all serovars have been associated with disease conditions (Gilmour and Gilmour, 1989). The disease potential of strains classified with the [*P.*] *aerogenes*-complex remains to be investigated in more detail (Bisgaard, 1993). Recent investigations (Kuhnert et al., 2000) showed that a new RTX operon, named *paxCABD* could be detected specifically in clinical isolates of [*P.*] *aerogenes*, which were all associated with abortion in pigs. Mice infected by [*P.*] *pneumotropica* developed bronchopneumonia, lobar pneumonia, or pleuropneumonia (Macy et al., 2000).

The mechanisms behind virulence and pathogenesis of fowl cholera remain unclear (Christensen and Bisgaard, 1997; Christensen and Bisgaard, 2000). Cloning of the toxin gene from toxigenic *P. multocida* into *Escherichia coli* has enabled a more detailed description of the pathogenesis of atrophic rhinitis (Kamps et al., 1990; Lax and Chanter, 1990; Petersen and Foged, 1990b). The toxin associated with atrophic rhinitis in pigs and other animals is encoded by the *toxA* gene (Petersen, 1990a). Sequencing of the entire genome of a common avian clone of *P. multocida* (May et al., 2001) has provided a basis for future research into virulence factors and mechanisms of pathogenesis including host specificity of certain disease aspects of this pathogen. Some virulence factors have been identified.

Purified LPS of *P. multocida* was found to cause suppurative airsacculitis, pleuritis and pneumonia in turkeys (Kunkle and Rimler, 1998). In addition, LPSs of *P. multocida* capsular type A were found to affect the humoral and cell-mediated immune response (Maslog et al., 1999).

## Antibiotic Sensitivity

Minimum inhibitory concentrations (MICs) of selected antimicrobial agents have been reviewed by Rimler and Rhoades (1989). Most human isolates of *Pasteurella* are susceptible to penicillin (Holst et al., 1992), the antibiotic of choice for local wound infections (Bisgaard et al., 1994). Other  $\beta$ -lactam antibiotics should be effective as well (Jorgensen et al., 1991). Macrolides and, especially for urinary and respiratory infections, fluoroquinolones, appear to be useful in human and animal infections (Gaillot et al., 1995; Hanan et al., 2000). [*Pasteurella*] *caballi* and [*P.*] *testudinis* have been found susceptible to ampicillin (Snipes and Biberstein, 1982; Schlater et al., 1989). Most strains are also susceptible to tetracyclines, but [*P.*] *testudinis* is resistant (Holst et al., 1992; Snipes and Biber-

stein, 1982). Marbofloxacin, a fluoroquinolone exclusively for veterinary use, might be useful in treatment of dogs and cats (Spreng et al., 1995). Tilcomycin, a macrolide antibiotic for use in veterinary medicine, proved to be effective in the treatment of pasteurellosis in rabbits (McKay et

al., 1996). Recent publications recommended treatment of animal infections with sulfonamides, tetracyclines, streptomycin and florfenicol (Kehrenberg et al., 2001b; Table 7). Laboratory mice might be treated for [*P.*] *pneumotropica* with enrofloxacin (Macy et al., 2000).

Table 7. Susceptibility and resistance to selected antibiotics.

Antibiotic agent	Species	Susceptibility	Resistance	References
<b>Penicillins</b>				
Amoxicillin	<i>P. multocida</i>	None	All	Rosenau et al., 1991
Ampicillin	<i>P. multocida</i>	Most	Few	McKay et al., 1996 Kehrenberg et al., 2001
Penicillin	<i>P. multocida</i>	Most	Some	McKay et al., 1996 Kehrenberg et al., 2001
Ticarcillin	[ <i>P.</i> ] <i>aerogenes</i>	Inactive	NI	Frederiksen, 1989
	<i>P. multocida</i>	None	All	Rosenau et al., 1991
	[ <i>P.</i> ] <i>aerogenes</i>	Inactive	NI	Frederiksen, 1989
<b>Cephalosporins</b>				
Cephalothin	[ <i>P.</i> ] <i>aerogenes</i>	Active	NI	Frederiksen, 1989
	<i>P. multocida</i>	Active	NI	Blackwood et al., 1996
Ceftriaxone	<i>P. multocida</i>	None	All	Rosenau et al., 1991
Ceftiofur	<i>P. multocida</i>	Active	NI	Boerlin et al., 2000
Cefquinome	<i>P. multocida</i>	Most	Few	Kehrenberg et al., 2001; Salmon et al., 1995
Macrolides	<i>P. multocida</i>	Most	Few	Kehrenberg et al., 2001
	Erythromycin	<i>P. multocida</i>	Inactive/Active	None
Tilcomycin	<i>P. multocida</i>	Active	None	McKay et al., 1996 Kehrenberg et al., 2001
Tylosin	<i>P. multocida</i>	Most	Some	Kehrenberg et al., 2001
<b>Aminoglycosides</b>				
Gentamycin	<i>P. multocida</i>	All	None	McKay et al., 1996 Snipes et al., 1989
Kanamycin	<i>P. multocida</i>	All	None	Snipes et al., 1989 Coté et al., 1991
	[ <i>P.</i> ] <i>aerogenes</i>	NI	One	Kehrenberg and Schwarz, 2000
Spectinomycin	<i>P. multocida</i>	All	None	Coté et al., 1991
Streptomycin	[ <i>P.</i> ] <i>aerogenes</i>	None	All	Kehrenberg and Schwarz, 2001
	<i>P. multocida</i>	Some	Some	McKay et al., 1996 Kehrenberg et al., 2001
<b>Tetracyclines</b>				
Minocycline	<i>P. multocida</i>	Some	One	Chaslus-Dancla et al., 1995
Tetracycline	[ <i>P.</i> ] <i>aerogenes</i>	None	All	Kehrenberg and Schwarz, 2001
	<i>P. multocida</i>	Most	Few	McKay et al., 1996 Kehrenberg et al., 2001
Sulfonamides	[ <i>P.</i> ] <i>aerogenes</i>	Some	Most	Kehrenberg and Schwarz, 2001
	<i>P. multocida</i>	Few	Most	McKay et al., 1996 Kehrenberg et al., 2001
Trimethoprim	[ <i>P.</i> ] <i>aerogenes</i>	Some	Most	Kehrenberg and Schwarz, 2001
	<i>P. multocida</i>	Most	Few	McKay et al., 1996 Kehrenberg et al., 2001
Chloramphenicol	[ <i>P.</i> ] <i>aerogenes</i>	Most	Some	Kehrenberg and Schwarz, 2001
	<i>P. multocida</i>	Few	Most	Kehrenberg et al., 2001
<b>Quinolones</b>				
Enrofloxacin	<i>P. multocida</i>	Most	Few	Kehrenberg et al., 2001; Salmon et al., 1995
Marbofloxacin	<i>P. multocida</i>	All	None	Spreng et al., 1995
Nalidixic acid	<i>P. multocida</i>	Few	Most	Kehrenberg et al., 2001
<b>Other compounds</b>				
Neomycin	<i>P. multocida</i>	All	None	McKay et al., 1996
Nitrofurantoin	<i>P. multocida</i>	All	None	McKay et al., 1996
Novobiocin	<i>P. multocida</i>	Active	NI	Morris et al., 1989
Spiramycin	<i>P. multocida</i>	Active	NI	Kehrenberg et al., 2001

Abbreviations: none, 0%; few, 1–33%; some, 34–66%; most 67–99%; all, 100% of strains; and NI, no information.

For feed medication of turkeys, chlortetracycline, furazolidone, penicillin, and neomycin-oxytetracycline might be used, while sulfadimethoxine, penicillin and tetracycline might be used for medication in water (Morris et al., 1989).

## Applications

Vaccines for prevention of diseases have only been developed for a few members of *Pasteurella* so far. Either live attenuated bacteria or bacterins have been used for vaccination (Confer, 1993). With live attenuated vaccines, immunity might be obtained against different serotypes. These vaccines, however, might revert to the virulent wild type or just become pathogenic under conditions such as stress or by immunosuppression (Morris et al., 1989).

Classical live vaccine strains include CU and M-9 strains against fowl cholera (e.g., Kim and Nagaraja, 1990). An interest in improving and developing more defined strains of *P. multocida* for use as live attenuated vaccines, however, is strongly needed (Boyce et al., 2000b). An *aroA* mutant strain, PMP3, of *P. multocida* was protective against homo- and heterologous challenge in a mouse model (Homchampa et al., 1997). Two *aroA* mutant strains, PMP1 and PMP3, were further tested in a chicken model where protection was achieved by intratracheal vaccination and challenge (Scott et al., 1999). Examples of live *P. multocida* vaccines of avian origin are Avichol, Orachol, PM one vax, M Nine Vax, M Nine Vax C (Schering-Plough Animal Health, USA), Choleravac-PM-1 (Intervet, USA) and ViClemcol-C (Vineland Laboratories, USA). The vaccine, Once PMH (Intervet), includes both modified avirulent *P. multocida* and *Mannheimia haemolytica*.

The alternative to live vaccines is to use killed bacteria or cellular components (bacterins). This approach excludes a disease risk through reversion to the virulent form, but these vaccines are normally only active against homologous serotypes and the vaccine normally has to be injected. A purified OMP of *P. multocida* was able to protect mice against homologous challenge with *P. multocida* (Ruffolo and Adler, 1996). In addition, it was found that OMPs expressed under iron-limiting conditions improved protection against heterologous challenge in mice compared to those expressed under normal iron concentrations (Ruffolo et al., 1998). Examples of bacterins of *P. multocida* are Inacti VACFC3 (Maine Biological Laboratories, USA) and PABAC (Fort Dodge Animal Health, USA). Presponse HM (Fort Dodge Laboratories) is a bacterin based

upon both *P. multocida* and *Mannheimia haemolytica*.

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## The Genus *Cardiobacterium*

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The genus *Cardiobacterium* consists of one species, *Cardiobacterium hominis*, originally designated “group II D organisms” until further described and named by Slotnick and Dougherty (1964). This organism is a fastidious, facultatively anaerobic, nonmotile, pleomorphic Gram-negative rod with a fermentative type of metabolism.

The first known report of these organisms was by Tucker et al. (1962). This paper described four cases of endocarditis caused by a *Pasteurella*-like organism. These isolates and two subsequent isolates, also from cases of endocarditis, were designated as “group II D” by the Centers for Disease Control, Atlanta, GA, until the name *C. hominis* was proposed by Slotnick and Dougherty (1964). The current assumption is that infectious endocarditis is the only disease caused by *C. hominis* in humans. This assumption may be valid or it may be based on a lack of more complete knowledge of the biology of the organism. Although past isolation of *C. hominis* has been almost exclusively limited to blood cultures, there have been at least two isolations from cerebral spinal fluid (Slotnick, 1968; Francioli et al., 1983).

Reference is not an exact match Slotnick (1964, 1968), in an attempt to extend our knowledge of the human occurrence of this organism, used fluorescent antibody smears and culture techniques to sample other sites. He and his colleagues isolated *C. hominis* from nose and throat specimens of 68 of 100 persons sampled, from cervical and vaginal cultures of 2 among 159 studied, and obtained positive fluorescent smears from stool specimens of 14 of 20 individuals. No positive stool cultures were obtained because of a lack of a selective medium and the overgrowth of *C. hominis* by the enteric bacilli. No isolations or positive smears were obtained from any urine specimens collected (no sample size was given for the urine specimens). These investigators concluded that *C. hominis* is part of

the indigenous commensal respiratory flora and supported this conclusion with the following observations: 1) None of the individuals harboring the organism had any signs or symptoms of disease. 2) Injection of viable suspensions of representative strains into laboratory animals elicited no visible sign of disease. 3) In relation to the high proportion of individuals who harbor the organism, few clinical cases occur. With the exception of these studies, there is a paucity of information concerning the ecology and pathogenicity of these organisms. All recently published information is limited to descriptive case reports of isolation of *C. hominis* from bacterial endocarditis, with the exception of one case report of *C. hominis* meningitis (Francioli et al., 1983). There appear to be no published reports on pathogenicity or virulence mechanisms.

Even though *C. hominis* was originally called a *Pasteurella*-like organism (Tucker et al., 1962), it was soon discovered that it was antigenically unrelated to members of the genus *Pasteurella* as well as to the genera *Brucella*, *Bordetella*, *Moraxella*, *Hemophilus*, *Streptobacillus*, *Corynebacterium*, *Bacteroides*, *Neisseria*, *Escherichia*, *Aerobacter* (*Enterobacter*) and *Lactobacillus* (Slotnick et al., 1964). It was compared to both Gram-negative and Gram-positive organisms because of the Gram-variable characteristics noted by early investigators. Because of this anomalous Gram-stain reaction, the fine structure of *C. hominis* was studied by Reyn et al. (1971). These investigators found that the cell wall was of the Gram-negative type, but an unusual feature of all strains examined was a 20–40-nm thick polar cap. This material was strictly limited to the terminal portion of the cell, adhering to the outside of the cell wall. Profile sections of this cap indicated that it was formed by radial arrangements of tufts of material but subsequent studies employing freeze-etching and negative-staining techniques were unable to further elucidate the nature of this cap material. The other notable feature of the *C. hominis* cell wall was an unusually dense outer layer. This layer was composed of a repeating structure which con-

\*This chapter was taken unchanged from the second edition.

Table 1. Biochemical differentiation of *Cardiobacterium hominis* and other fastidious Gram-negative rods.

	Fermentation of:									
	CAT	OXI	IND	NIT	GLU	LAC	MAL	MAN	SUC	XYL
<i>Cardiobacterium hominis</i>	-	+	+	-	+	-	+	+	+	-
<i>Kingella</i> species	-	+	D	D	+	-	D	-	D	-
<i>Capnocytophaga</i> species (DF-1, DF-2)	D	D	-	D	+	+	D	-	D	-
<i>Actinobacillus actinomycetemcomitans</i>	+	D	-	+	+	-	+	+	-	D
<i>Pasteurella</i> species	+	+	D	+	+	D	D	D	+	D
<i>Eikenella corrodens</i>	-	+	-	+	-	-	-	-	-	-
<i>Haemophilus aphrophilus</i>	-	D	-	+	+	+	+	-	+	-
<i>Streptobacillus moniliformis</i>	-	-	-	-	+	D	+	-	-	-
<i>Brucella</i> species	+	+	-	+	-	-	-	-	-	-
<i>Bordetella</i> species	+	-	-	+	-	-	-	-	-	-

<sup>a</sup>CAT = catalase; OXI = oxidase; IND = indole; NIT = nitrate reduction; GLU = glucose; LAC = lactose; MAL = maltose; MAN = mannitol; SUC = sucrose; XYL = xylose; D = different biotypes.

sisted of units exhibiting tetragonal or rectangular packing. The periodicity of these arrays measured 5.5 nm; the average diameter of the units was 3.4 nm and the space between them was 2 nm. Surface arrays are more typical of Gram-positive bacteria than of Gram-negative bacteria but are not unknown in the latter (Reyn et al., 1971).

Limited genetic studies have demonstrated that the GC content of the DNA for *C. hominis* is quite different from the other Gram-negative rod-shaped organisms which have similar physiological characteristics. *C. hominis* GC is 59–60%, *Haemophilus aphrophilus* is 42%, *Kingella* sp. is 47.3–54.8%, *Actinobacillus actinomycetemcomitans* is 42.7%, *Pasteurella* sp. is 40–45%, *Eikenella corrodens* is 56.2–58.2%, and *Capnocytophaga* sp. is 33–41% (Weaver, 1984).

## Isolation

Except for the studies of Slotnick and colleagues, all published isolation attempts have been from normally sterile sites, predominantly blood. The one recorded isolation from cerebral spinal fluid does not give cultural information (Francioli et al., 1983). *C. hominis*, although a slow-growing organism, appears to be able to initiate growth on a variety of blood culture media under both anaerobic and aerobic conditions. Only two types of basal media were reported not to support primary blood culture isolation attempts—thioglycollate and Schaedler broths (Bruun et al., 1983; Midgley et al., 1970; Piot et al., 1978). Since it has been reported that *C. hominis* growth may not be noted until after 23 days of incubation, the failure to see growth in the above two media may be due to the fact that the cultures were discarded as negative after 14 days of incubation (Geraci et al., 1978). More typically, growth of the organism is seen after 5 to 8 days of incubation. For subculture from primary cul-

Table 2. Biochemical characteristics of *Cardiobacterium hominis*.

Characteristic	Reaction
Oxidase	+
Catalase	-
Motility	-
Nitrate reduction	-
Indole	+
Urease	-
Citrate	-
Esculin hydrolysis	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Arginine dihydrolase	-
o-Nitrophenyl-β-D-galactopyranoside (ONPG)	-
Growth on MacConkey agar	-
Gelatin liquefaction	D <sup>a</sup>
Litmus milk acidification	D
Methyl red	D
Voges-Proskauer	-
Tween 20 hydrolysis	-
Tween 40 hydrolysis	-
Acid from:	
Glucose	+
Adonitol	-
Arabinose	-
Cellobiose	-
Dulcitol	-
Erythritol	-
Fructose	+
Galactose	-
Inositol	-
Lactose	-
Maltose	+
Mannitol	+
Mannose	+
Melezitose	-
Melibiose	-
Rhamnose	-
Salicin	-
Sorbitol	+
Sucrose	+
Trehalose	-
Xylose	-

D = different biotypes.

tures, 5% sheep blood or chocolate agar are most frequently used although *C. hominis* will grow on media without blood (Midgley et al., 1970). Neither CO<sub>2</sub> or humidity is an absolute growth requirement for all strains, but both supplemental CO<sub>2</sub> and increased humidity enhance growth (Savage et al., 1977).

For isolation from nonsterile sites, no selective medium is presently available. Earlier studies on throat and nose specimens used trypticase-soy agar slants or plates enriched with 5% human blood incubated at 37°C for 48–72 h (Slotnick et al., 1964). Slotnick's subsequent screen of vaginal and cervical swabs for *C. hominis* employed a slightly different method. Swabs were placed in tubes containing 2 ml of Trypticase Soy Broth (BBL) for transport to the laboratory where they were plated on a Casman's blood agar plate and a chocolate agar plate (BBL). Incubation was at 37°C for 4 days in a candle jar (Slotnick, 1968).

## Identification

Differential characteristics of *C. hominis* which distinguish it from other similar Gram-negative organisms are given in Table 1. General biochemical characteristics of *C. hominis* are given in Table 2.

Microscopically, cells of *C. hominis* are pleomorphic Gram-negative rods which may show retention of crystal violet in the central portion or in the enlarged ends. Teardrop cells and filaments of varying lengths may occur. One study attributes this extreme pleomorphism and the irregular staining to growth on media without yeast extract. This report states that when *C. hominis* was grown on a medium containing yeast extract, the organisms appeared mainly as uniform Gram-negative rods in contrast to the pleomorphism and Gram-variable characteris-

tics exhibited on media without yeast (Savage et al., 1977).

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## The Genus *Actinobacillus*

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### Introduction

Members of the genus *Actinobacillus* are small, Gram-negative, pleomorphic, coccobacillary rods that are facultatively anaerobic, indole negative, -galactosidase and urease positive and reduce nitrates (Olsen and Møller, 2004a). Apart from *A. pleuropneumoniae* and some *A. suis* strains, most *Actinobacillus* isolates grow on MacConkey agar. The optimum temperature for growth is 37°C and all have complex nutritional requirements. These bacteria are always associated with mucous membranes and the host range of *Actinobacillus* spp. tends to be limited. Although members of the genus *Actinobacillus* can be benign commensals of the respiratory, alimentary, and genital tracts, *Actinobacillus equuli*, *A. lignieresii*, *A. pleuropneumoniae* and *A. suis* can cause important diseases in domestic animals. (*Actinobacillus actinomycescomitans* is the only member of the genus that routinely colonizes humans; *A. hominus* and *A. ureae* are associated with rare opportunistic infections in compromised individuals.

### Phylogeny

When the family Pasteurellaceae was first described in 1981 by Pohl et al., it was comprised of three genera: *Pasteurella*, *Haemophilus* and *Actinobacillus*. Since that time it has undergone considerable revision and two new genera, *Lonepinella* and *Mannheimia* (formerly *Pasteurella* complex) have been added (Osawa et al., 1996; Angen et al., 1999). Recent 16S rRNA studies of more than 120 strains of the family Pasteurellaceae revealed 20 genus level groups (I. Olsen et al., manuscript in press). On the basis of this analysis, the *Actinobacillus* group is comprised of *A. suis*, *A. equuli*, *A. hominis*, *A. ureae*, *A. lignieresii* and *A. pleuropneumoniae*. In addition, several other species/strains including *H. parahaemolyticus*, *Actinobacillus* spp. CCUG 19799 and CCUG 28015R, Bisgaard's taxon 8 CCUG 16494, Bisgaard's taxon 9 CCUG 24862, *Actinobacillus (capsulatus)* MCCM 00149 and *H. parainfluenzae* belong to the group *Actinobacil-*

*lus minor*, *A. porcinus*, *A. rossii* and *A. indolicus* are closely related to the *Actinobacillus* group while (*A.*) *capsulatus*, *A. muris*, *A. seminis* and (*A.*) *actinomycescomitans* are only distantly related. When 16S RNA, sequence data, DNA–DNA hybridizations, and phenotypic analysis are considered together, Christensen and Bisgaard (2004) suggest that *Actinobacillus sensu stricto* should be limited to *A. lignieresii*, *A. pleuropneumoniae*, *A. equuli* subsp. *equuli*, *A. equuli* subsp. *haemolyticus*, (taxon 11 of Bisgaard), *A. hominis*, *A. suis*, *A. ureae*, *A. arthritidis* (taxon 9 of Bisgaard), *Actinobacillus* genomospecies 1 and 2 and taxa 8 and 26 of Bisgaard. Further studies using additional strains and other molecular techniques are needed to further clarify the phylogenetic relationships within the genus *Actinobacillus*, but division of the family Pasteurellaceae, and even genera within the family, into phylogenically and phenotypically coherent groups may remain problematic.

### Taxonomy

In the ninth edition of *Bergey's Manual of Determinative Bacteriology*, the genus *Actinobacillus* included the 11 species: (*A.*) *actinomycescomitans*, (*A.*) *capsulatus*, *A. equuli*, *A. hominis*, *A. lignieresii*, (*A.*) *muris*, *A. pleuropneumoniae*, (*A.*) *rossii*, (*A.*) *seminis*, *A. suis* and *A. ureae*. Four additional species, (*A.*) *delphinicola*, (*A.*) *minor*, (*A.*) *porcinus* and (*A.*) *indolicus* were later proposed (Foster et al., 1996; Møller et al., 1996). On the basis of DNA–DNA hybridization and 16S rRNA sequencing, there is a strong case for the inclusion of *A. equuli*, *A. hominis*, *A. lignieresii* and *A. pleuropneumoniae*, *A. suis* and *A. ureae*, but as noted below in this section, the taxonomic positions of isolates of many of these species is complicated by numerous overlapping genotypic and phenotypic features.

Determining the taxonomic position of (*A.*) *actinomycescomitans* is confusing, at best, for it appears that (*A.*) *actinomycescomitans* is more closely related to *Haemophilus aphrophilus* and *H. paraphrophilus* than to the type species of the genus *Actinobacillus*, *A. lignieresii*.



Further, it has been suggested that *H. aphrophilus* and *H. paraphrophilus* are not true *Haemophilus* spp. as they do not require X and/or V factor for growth (Kilian, 1976; Olsen and Møller, 2004a). Previous attempts to reclassify (*A.*) *actinomycetemcomitans* have not met with approval, and in the future, (*A.*) *actinomycetemcomitans* will likely be placed in a new genus, which may contain one or more subspecies or even species.

At this point, the taxonomic positions of *A. equuli*, *A. lignieresii* and *A. suis* also seem to be very problematic. In 2002, Christensen et al. (2002b) proposed a new classification scheme for equine isolates of *A. equuli*, which had previously been reported as *Actinobacillus equuli*, variants of *A. equuli*, *Actinobacillus suis*, *A. suis*-like, or Bisgaard taxon 11. On the basis of 16S rRNA and DNA-DNA hybridization studies, they recommended that these organisms be classified as *A. equuli* subsp. *equuli* subsp. nov. and *A. equuli* subsp. *haemolyticus* subsp. nov. The picture has been further clarified by Berthoud et al. (2002), who found that two 16S rRNA groups could be identified and that all hemolytic strains of *A. equuli* contained the *aqx* operon. The presence of the *aqx* operon could not, however, be correlated with either of the phylogenetic groups. Kuhnert et al. (2003) further examined representative *A. suis* and *A. equuli* and concluded that the only way that *A. equuli* and *A. suis* can be differentiated is on the basis of their RTX (repeats-in-toxin) toxins. The inter-relatedness of these organisms has been further demonstrated in recent studies by Christensen et al. (2002a) of equine strains of *A. lignieresii*. They found that these organisms are genetically most closely related to Bisgaard taxon 11 and *A. equuli* subsp. *haemolyticus*. Since no distinguishing phenotypic characteristics allow them to be separated from *A. lignieresii*, they have been designated “*Actinobacillus* genomospecies 1.”

*Haemophilus pleuropneumoniae* was first described in detail by Shope et al. in 1964. Prior to that, organisms causing the same disease had been called “*Haemophilus parainfluenzae*” or “*Haemophilus parahaemolytica*” (Taylor, 1999a). It was finally placed in the genus *Actinobacillus* by Pohl et al. in 1983. On the basis of DNA-DNA hybridization studies, it is clearly a member of the genus. Indeed, the serotype 1 strain (Shope 4044) shares over 70% homology with *A. lignieresii* making it technically a member of the same species (Borr et al., 1991).

## Habitat

Members of the genus *Actinobacillus* are generally found as parasites or species-specific com-

mensals of the upper respiratory, alimentary and genital tracts of domestic animals including cattle, sheep, horses, swine, nonhuman primates and man. Although there have been few systematic studies, survival of these organisms in the environment is thought to be poor, and there is no evidence (other than [*A.*] *actinomycetemcomitans*, which can be cultured from Old and New World monkeys and great apes) that they can replicate outside their respective hosts.

(*Actinobacillus*) *actinomycetemcomitans* is a pioneer colonizer of the upper aerodigestive tract of both humans and nonhuman primates where acquisition of the organism is seen at 3–6 months on the tongue and oral mucosa in both humans (Lamell et al., 2000) and captive *M. fascicularis* (macaque monkeys; Beighton et al., 1989). The organism has been isolated from patients with infections of the heart, urinary tract and brain. The bacterium is a member of the HACEK (*Hemophilus* spp., *Actinobacillus* spp., *Cardiobacterium* spp., *Eikenella* spp., *Kingella* spp.) group, which is the most common cause of Gram-negative endocarditis in children (Meyer, 1989; Das et al., 1997). The most studied infection by this organism is localized juvenile periodontitis (LJP; Zambon, 1985) recently renamed “localized aggressive periodontitis,” LAP, a destructive disease of the periodontal ligament that affects the first molars and central incisors and results in rapid destruction of alveolar bone and subsequent tooth loss (Armitage, 1999).

*Actinobacillus ureae* is an uncommon inhabitant of the upper respiratory tract of humans that is associated with rhinitis, sinusitis and chronic bronchitis (Henriksen and Jyssum, 1960). Cases of meningitis, pneumonia, peritonitis and sepsis also have been attributed to this organism. The organism is thought to be a commensal, and the association of *A. ureae* meningitis with skull and facial fractures or subsequent surgery suggests that trauma may create an avenue for infection by this otherwise benign organism (for review, see Kingsland and Guss, 1995).

*Actinobacillus hominis* is another uncommon commensal of the human upper aerodigestive tract. Originally described as an “*A. ureae*-like” bacterium (Henriksen and Jyssum, 1960), it was later recognized as a distinct species (Friis-Møller, 1981; Friis-Møller et al., 2001). This organism has a predilection for infection of patients with pre-existing medical conditions such as chronic alcoholism, cardiovascular disease, cancers of the aerodigestive tract, and drug addiction.

*Actinobacillus equuli* is the causative agent of “sleepy foal disease” and “joint ill” in young horses and is occasionally associated with diseases in older horses (Phillips, 1992; Quinn et al., 2002). It is a common resident of the oral cavity

and upper respiratory tract of adult horses (Sternberg and Brändström, 1999). It has also been recovered from feces of healthy horses (Phillips, 1992) and enteric lesions (Al-Mashat et al., 1986). *Actinobacillus equuli* can be transmitted from the mare to the foal via the oral, respiratory, or the umbilical route at or following birth. Although there have been reports of sporadic cases of *A. equuli* in swine (Windsor, 1973) and other species, its presence in healthy animals other than horses has not been documented.

*Actinobacillus lignieresii* is a common commensal of the mouth and rumen of healthy cattle and sheep. It is the causative agent of "wooden tongue," a chronic disease of the soft tissues of the head and neck characterized by granulomatous lesions, which superficially resemble those of *Actinomyces bovis* (Quinn et al., 2002). Although the presence of *A. lignieresii* has been reported in other species, it would appear that these organisms were other Pasteurellaceae (Bisgaard et al., 1986; Christensen et al., 2002a).

*Actinobacillus pleuropneumoniae*, in contrast to other *Actinobacillus* spp., often behaves as a primary pathogen. Depending on the serovar and strain of the bacterium and the immune status of the infected animal, *A. pleuropneumoniae* can cause a rapidly fatal and highly contagious acute pleuropneumonia (Sebunya and Saunders, 1983). Animals that survive acute infection may continue to harbor the organism in their tonsils, nasal cavities, and lung lesions (Møller and Kilian, 1990; Taylor, 1999a). Less virulent strains may persist in herds without causing any apparent problems until the animals are subjected to stress (Rosendal and Mitchell, 1983; Taylor, 1999a). Transmission of *A. pleuropneumoniae* by aerosol droplets over short distances or through direct contact with infected pigs has been demonstrated experimentally and in the field (Chiers et al., 2002). Airborne transmission from farm to farm is still a matter of debate, but there is growing evidence that this can occur (Fussing et al., 1998; Taylor, 1999a). Survival outside of the host is considered to be of short duration, but it may last for several days at cool temperatures in organic matter. It has also been reported to survive for 30 days in clean water at 4°C (Taylor, 1999a). Although mice can be experimentally infected, there is no evidence for carriage in animals other than swine (Sebunya and Saunders, 1982).

*Actinobacillus suis* is an early colonizer of the upper respiratory tract of swine, and it has been reported to be isolated from the genital tract of healthy sows (MacInnes and Desrosiers, 1999; Taylor, 1999b). Traditionally, *A. suis* was associated with the sporadic cases of septicemia and sudden death in young piglets, but more recently

it has been associated with a wide range of clinical conditions (including erysipelas-like lesions, arthritis, pneumonia, metritis, abortion and septicemia) in older animals in high health status herds. Infection with *A. suis* likely occurs via invasion of the upper respiratory tract or less commonly via direct entry to the bloodstream, e.g., through abrasion of the skin or umbilical lesions. Since its original description in 1962, sporadic cases of *Actinobacillus suis* or *A. suis*-like organisms have been reported in a variety of birds and mammals (Phillips, 1984). Although recent studies suggest that many strains from non-porcine sources may not have been *A. suis* sensu stricto; there is good evidence that at least some strains of *A. suis* can infect mammals other than swine (Christensen et al., 2002b; Jeannotte et al., 2002). Despite the ability to cause sporadic disease in other species, no reports have suggested that *A. suis* has a normal habitat outside of swine.

## Isolation

Most isolates of the genus *Actinobacillus* can be easily cultured on rich media such as sheep blood, chocolate blood, PPLO (pleuropneumonia-like organism), or brain heart infusion agar. *Actinobacillus suis* and *A. pleuropneumoniae* (with the addition of nicotinamide adenine dinucleotide [NAD]) will generally grow on somewhat simpler substrates such as tryptone-yeast extract (TYE) medium (O'Reilly and Niven, 1986). Some strains of *A. pleuropneumoniae* will grow on Herriott's minimal medium, but not well (Herriott et al., 1970). Selective media such as Phillips selective medium for *A. lignieresii* are needed to isolate *Actinobacillus* from environments with mixed bacterial populations. TSVB agar (40 g of tryptic soy agar, 1 g of yeast extract, 100 ml of horse serum, 75 mg of bacitracin, and 5 mg of vancomycin per liter of agar) is an excellent primary selective medium for (*A. actinomycetemcomitans* that can be used to detect the microorganism in levels as low as 20 viable cells per liter (Slots et al., 1982). Jacobsen and Nielsen (1995) tested four different selective media and found that S-MBA (selective meat and blood agar) containing bacitracin (100 µg/ml), lincomycin (1 µg/ml), crystal violet (1 µg/ml), and nystatin (50 µg/ml) to be most effective for the isolation of *A. pleuropneumoniae* from pig tonsil. Columbia agar supplemented with 3% horse serum, 5% yeast extract, and these same antibiotics has also been used (Chiers et al., 2002). Clindamycin (0.5 µg/ml) has been used to improve the isolation of *A. equuli* from nonsterile sites (Sternberg and Brändström, 1999).

## Identification

Members of the genus *Actinobacillus* are small, nonmotile, nonsporulating, pleomorphic rods (~0.3–0.5 × 0.6–1.4 μm). Depending on the growth conditions, short bacillary or coccobacillary rods may be interspersed with coccocellular elements giving rise to a “Morse code” appearance. Cells are Gram negative, but staining may be irregular. On media containing glucose or maltose, long rods up to 6 μm may be seen. Colonies are small (1–2 mm) and usually sticky especially on primary isolation, but they often lose this property on subculture. All actinobacilli reduce nitrate, produce alkaline phosphatase, urease (except [*A.*] *actinomycetemcomitans*), and acid from maltose (Table 1). Members of the genus are indole-, ornithine decarboxylase-, and sorbitol-negative. Growth is improved in an atmosphere of 5% CO<sub>2</sub>. A number of tests used for differentiating *Actinobacillus* from other members of the family Pasteurellaceae have been described by Olsen et al. (2004b; Table 2), but given the overlap in phenotypic properties, identification to species or even to the genus level is sometimes difficult within this family. (*Actinobacillus*) *actinomycetemcomitans*

This organism can be isolated in the gingival crevice and tonsillar crypts. When grown on TSBV agar, the colonies display a classic star-shaped inner structure. The organism does not produce alkaline phosphatase or urease, making

it quite distinct from other actinobacilli (Fig. 1A, Table 2).

### *Actinobacillus equuli*

Usually isolated from healthy or diseased horses, *Actinobacillus equuli* can be difficult to identify, as phenotypic properties such as hemolysis and the ability to ferment arabinose are variable (Kuhnert et al., 2003). Growth is seen on MacConkey agar. When first isolated, colonies (which are round and grayish) may be rough but become smooth upon subculture. Broth cultures may be extremely viscous. *Actinobacillus equuli* ferments glucose, dextrose, xylose, mannitol, lactose, sucrose, maltose, raffinose, melibiose, and trehalose but not arabinose. It is nitrate-, oxidase- and urease-positive but indole- and esculin-negative (Table 2). Differential identification of hemolytic *A. equuli* from *A. suis* can be done on the basis of esculin hydrolysis and arabinose fermentation reactions. These organisms can also be differentiated on the basis of their pattern of RTX toxins (Table 3). Growth typically occurs at 20–39°C, but some strains will grow at 44°C.

### *Actinobacillus lignieresii*

This organism is usually isolated from characteristic lesions of diseased cattle or sheep. Colonies are usually sticky upon first isolation but lose this

Table 1. Differential characteristics for members of the family Pasteurellaceae.

Characteristics	<i>Haemophilus</i> <sup>a</sup>	<i>Actinobacillus</i> <sup>a</sup>	<i>Pasteurella</i> <sup>a</sup>	<i>Lonepinella</i> <sup>b</sup>	<i>Mannheimia</i> <sup>c</sup>
Sticky colonies	–	d	–	U	–
Hemolysis SBA	d	d	–	–	d
V factor-dependency	+	– <sup>d</sup>	–	–	–
X factor-dependency	+	–	–	–	–
Urease	d	+	– <sup>f</sup>	–	–
Ornithine decarboxylase	– <sup>e</sup>	–	d	–	d
Indole	d	–	+	–	d
α-Fucosidase	–	d	–	U	d
Acid production from					
D-Glucose	+	+	+	+	+
D-Mannitol	–	+ <sup>g</sup>	d	–	+
D-Mannose	–	d	+	U	–
Melibiose	–	d	–	d	d
<i>i</i> -Inositol	–	d	–	–	d
D-Sorbitol	–	d	d	U	d
Trehalose	–	d	d	U	–

Symbols and abbreviations: +, 90–100%; –, 0–10%; d, variable; U, unknown; and SBA, sheep blood agar.

<sup>a</sup>Sensu stricto species only.

<sup>b</sup>Osawa et al. (1995).

<sup>c</sup>Angen et al. (1999).

<sup>d</sup>Some strains of *A. pleuropneumoniae* are positive.

<sup>e</sup>Some strains of *H. influenzae* are positive.

<sup>f</sup>*Pasteurella dagmatis* is positive.

<sup>g</sup>*Actinobacillus suis* is negative.

Table 2. Differential characteristics of *Actinobacillus*. Reproduced with permission from Olson et al., 2004b.

Reaction	<i>A. acinetomyces-temcomitans</i>	<i>A. ureae</i>	<i>A. hominis</i>	<i>A. equuli</i>	<i>A. lignieresii</i>	<i>A. pleuropneumoniae</i>	<i>A. suis</i>
NAD requirement	–	–	–	–	–	+ <sup>a</sup>	–
Hemolysis on sheep blood agar	–	–	ND	V	–	+ <sup>b</sup>	+
CAMP test with <i>S. aureus</i>	ND	ND	ND	–	–	+	–
Growth on MacConkey agar	–	–	ND	+	+	–	V
Nitrate	+	+	ND	+	+	+	+
Oxidase	+	(+)	+	+	+	V	+
Catalase	+	V	+	V	+	+	+
Indole	–	–	–	–	–	–	–
Alkaline phosphatase	+	+	+	+	+	+	+
Ornithine decarboxylase	–	–	–	–	–	–	–
Esculin hydrolysis	–	–	V	–	–	–	+
Urease	–	+	+	+	+	+	+
ONPG	–	–	+	+	V	+	+
Acid from							
(+)-L-Arabinose	–	–	–	–	V	–	+
Cellobiose	–	–	–	–	–	–	+
Galactose	+	–	+	V	V	V	V
Lactose	–	–	+	+	V	(+)	(+)
Maltose	+	+	+	+	+	V	+
Mannitol	+	+	+	+	+	+	–
Mannose	V	V	V	+	+	+	+
Melibiose	–	–	(+)	+	–	V	+
Raffinose	–	–	+	+	V	V	+
Salicin	–	–	V	–	–	–	+
Sorbitol	–	–	–	–	–	–	–
Sucrose	–	+	+	+	+	+	+
Trehalose	–	–	+	(+)	–	–	+
Xylose	+	–	+	+	(+)	+	+

Symbols: +, positive reaction; (+), delayed reaction, –, negative negative; and V, variable.

Abbreviations: NAD, nicotinamide adenine dinucleotide; CAMP, Christie, Atkins, Munch-Peterson (a test for co-hemolytic activity); ONPG, 2-nitrophenyl-β-D-galactopyranoside; and ND, not determined.

<sup>a</sup>Biotype 2 is NAD independent.

<sup>b</sup>In the presence of NAD.

From Weyant et al. (1996), Mutter (1999), Rycroft and Garside (2000), Quinn et al. (2002), I. Olsen et al. (manuscript in press), Christensen and Bisgaard, 2004

property with subculture. Small glistening colonies develop in 24 hours that are at first pale gray but later become pinkish. Broth cultures are turbid with little deposit. Growth is seen on both blood and MacConkey agar. *Actinobacillus lignieresii* ferments glucose, xylose, mannitol, lactose (late), sucrose, and maltose but not trehalose or melibiose. It is oxidase-, nitrate- and urease-positive but indole- and esculin-negative (Table 2). Growth occurs at 20–39°C; no growth is seen at 44°C.

Phillips Selective Medium for *Actinobacillus lignieresii* (Phillips, 1961; Phillips, 1964)

Phillips\* selective medium incorporates an antifungal agent and is prepared as follows:

Prepare a stock solution of oleandomycin phosphate (5 mg/ml) in sterile distilled water, distribute it in 0.2 ml amounts, and store it frozen at –20°C. After thawing, prepare a working solution by adding 9.8 ml of sterile distilled water. Prepare a stock suspension of

nystatin (20,000 units/ml) in sterile distilled water, distribute in 1-ml amounts, and store frozen at –20°C. Both these stocks will store satisfactorily for at least 2 months. Prepare the final medium by adding the antibiotics to horse blood agar to give final concentrations of oleandomycin (1 µg/ml) and nystatin (200 units/ml) as follows:

Hartley's digest agar (see below)	93 ml
Horse blood (oxalated)	5 ml
Oleandomycin phosphate working solution	1 ml
Nystatin stock suspension	1 ml

Melt the agar base and cool to 50°C. Add the blood and antibiotics and pour into sterile Petri plates.

Hartley's Digest Broth (Phillips, 1992)

Ox heart (minced)	3000 g
Distilled water	5 liters
Na <sub>2</sub> CO <sub>3</sub> (anhydrous; 0.8% solution)	5 liters
Pancreatin	50 g
HCl (concentrated)	80 ml

Table 3. RTX toxins in *Actinobacillus*.

Operon	Protein, function	Distribution
<i>apxI</i>	C	Activator of ApxI toxin
	A	105–110-kDa toxin; strong hemolytic and cytotoxic activities
	B	Transport proteins for ApxIA and ApxIIA
	D	
<i>apxII</i>	C	Activator of ApxII toxin
	A	103–105-kDa toxin; weak hemolytic and no cytotoxic activity
<i>apxIII</i>	C	Activator of ApxIII toxin
	A	120-kDa toxin; no hemolytic but strong cytotoxic activity
	B	Transport proteins for ApxIII
	D	
<i>apxIV</i>	ORF1	Required for ApxIVA activity
	A	200 kDa expressed only in vivo; weak hemolytic activity (+ CAMP reaction)
<i>aqx</i>	C	Activator of AqxA
	A	110kDa; strong hemolytic activity, + CAMP reaction
	B	Transport proteins for AqxA
	D	
<i>ltx</i>	C	Activator of LtxA activity
	A	116-kDa cytotoxin
	B	Transport proteins for LktA
	D	

Abbreviation: CAMP, Christie, Atkins, Munch-Peterson (a test for co-hemolytic activity).

From data in Brogan et al. (1994), Frey (1995), van Ostaaijen et al. (1997), Schaller et al. (1999), and Berthoud et al. (2002).

Mix the minced meat and water and heat to 80°C. Add the sodium carbonate and cool to 45°C. Add pancreatin and incubate at 45°C for 4 h, stirring frequently. When digestion is complete, add the hydrochloric acid and steam at 100°C for 30 min. Cool to room temperature and add 1 N NaOH (caustic soda) to bring the pH to 8.0. Boil for 25 min to precipitate phosphates and filter while hot. Allow to cool and adjust to pH 7.5. Sterilize by autoclaving at 121°C for 15 min. Prepare horse blood agar by adding 1% agar (Oxoid no. 1) to the digest broth, autoclave to sterilize, cool to 50°C and add 5% oxalated horse blood. Distribute into sterile Petri plates.

### *Actinobacillus pleuropneumoniae*

This bacterium is virtually always isolated from swine either from the lungs or nasal secretions of animals with signs of pleuropneumonia or from the tonsils of apparently healthy animals. Upon primary isolation on blood agar plates with a *Staphylococcus aureus* feed streak, the colonies are small (0.5–1 mm) and surrounded by a zone of  $\beta$ -hemolysis. Colonies, which are grayish white, may be of a waxy or soft glistening type (Fig. 1B). No growth occurs on MacConkey agar. Most strains require NAD (biotype 1) and form satellite colonies immediately adjacent to the feeder streak. *Actinobacillus pleuropneumoniae* ferments glucose, xylose, mannitol, lactose (late), and sucrose but not trehalose or arabinose. It is CAMP- (Christie, Atkins, Munch-Peterson), oxidase-, nitrate-, and urease-positive but indole-

and esculin-negative (Table 2). Differentiation of NAD-independent *A. pleuropneumoniae* from *A. suis* can be done on the basis of mannitol and arabinose reactions and RTX toxin profiles. Growth occurs at 20–39°C but not at 44°C.

#### Media for the Culture of *Actinobacillus pleuropneumoniae*

##### 5% Sheep Blood Agar with NAD

Dissolve 44 g of Columbia agar base (Difco) and qs to 1000 ml with double distilled H<sub>2</sub>O. Autoclave and cool to 45°C. Add 50 ml of defibrinated sheep blood and 10 ml of 10% (w/v) filter sterilized NAD.

##### Chocolate Blood Agar with NAD

Dissolve 44 g of Columbia agar base (Difco) and qs to 1000 ml with double distilled H<sub>2</sub>O.

Autoclave and cool to 55°C. Add 50 ml of defibrinated sheep blood, heat to 65°C for 30 min, cool to 45°C, and then add 1 ml of 10% (w/v) filter sterilized NAD.

##### TYE (O'Reilly and Niven, 1986)

Tryptone (Difco)	20 g
Yeast extract (Difco)	5 g
NaCl (10 mM)	5.84 g
KCl (10 mM)	0.75 g
Na <sub>2</sub> HPO <sub>4</sub> (10 mM)	1.42 g
KH <sub>2</sub> PO <sub>4</sub>	0.46 g
Glucose (10 mM)	1.80 g
Distilled H <sub>2</sub> O	950 ml

Adjust to pH 7.4 with KOH (not NaOH). Dilute up to 1000 ml with distilled H<sub>2</sub>O. Autoclave and cool to 45°C. Add 1 ml of 10% (w/v) filter sterilized NAD.

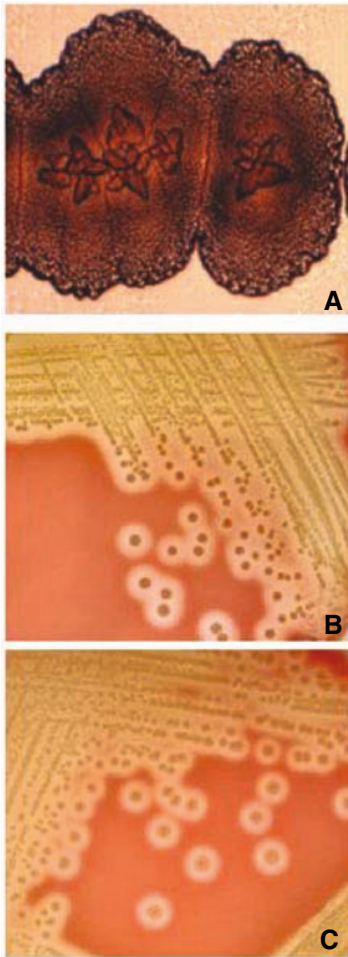


Fig. 1. Colonial appearance of actinobacilli on various agar media. A) *Actinobacillus acetomycetemcomitans* on TSVB agar. Courtesy of Dr. Cherng-Hsiung Lai, University of Pennsylvania. B) *A. pleuropneumoniae* CM5 on sheep blood agar. C) *A. suis* on sheep blood agar.

### *Actinobacillus suis*

This species can be isolated from tonsils of healthy swine and from the tissues of sick pigs. Colonies are usually sticky on first isolation and this property increases with prolonged incubation. Older colonies may develop a transparent border giving a “fried egg” appearance. Broth cultures may be viscous. On blood agar, *A. suis* colonies, which are creamy yellow, show  $\beta$ -hemolytic activity (Fig. 1C), but it is usually not as strong as that of *A. pleuropneumoniae* (Van Ostaaen et al., 1997). Some but not all strains will grow on MacConkey agar. *Actinobacillus suis* ferments glucose, xylose, lactose (late), sucrose, maltose, D-mannose, and trehalose but not mannitol. *Actinobacillus suis* are esculin-, nitrate- and urease-positive but indole-negative (Table 2). The optimum temperature for growth is 37°C.

## Preservation

Actinobacilli generally lose their viability after ~5 days of culture on solid media. They can survive for years at  $-80^{\circ}\text{C}$  or following lyophilization in various substrates including sterile rabbit serum, 20% peptone, or skim milk with 30% glycerol. Fifty percent survival of *A. pleuropneumoniae* stored at  $4^{\circ}\text{C}$  or at room temperature for almost a month in horse serum plus 0.01% NAD has also been reported (del Rio et al., 2003).

## Genetics

Studies of members of the genus *Actinobacillus* have been frustrated by their complex nutritional needs and, until recently, the absence of robust genetic tools (Frey and MacInnes, 1995b). Barriers to the use of “conventional” genetic tools such as Tn5 would seem to include powerful restriction systems and poor expression of selectable markers and other genes. It is not clear whether poor expression is a function of codon usage or failure to recognize promoters from heterologous systems. In recent years, a number of shuttle plasmids have been developed for work with various actinobacilli and other Pasteurellaceae (Table 4), but considerable strain-to-strain variation is seen when working with these vectors. The suicide conjugative plasmid, pLOF/Km, carrying a mini-Tn10 with an isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-inducible transposase located outside the mobile element has proven to be a very useful tool for the generation of defined mutations in *A. pleuropneumoniae* (Tascon et al., 1993). Single-step transconjugation systems were developed for the introduction of unmarked deletions into *A. pleuropneumoniae* (Oswald et al., 1999; Baltes et al., 2003). Although both of these systems were designed for use in *A. pleuropneumoniae*, they also work well in *A. suis* and could conceivably work in other *Actinobacillus* spp.

### (*Actinobacillus*) *actinomycetemcomitans*

The maintenance of DNA in plasmid form is rare in (*A.*) *actinomycetemcomitans* and posed an initial obstacle because isolation of a plasmid from (*A.*) *actinomycetemcomitans* was a necessary step to construct the intergeneric plasmids needed for various genetic transfer systems. Plasmids of various molecular sizes (4–20 kDa) were initially reported in 10 (*A.*) *actinomycetemcomitans* clinical isolates derived from periodontal lesions of patients with rapidly destructive periodontitis (Olsvik and Preus, 1989). All ten isolates had identical plasmid profiles composed of

Table 4. Useful vectors for the study of *Actinobacillus*.

Plasmid	Properties, origin	Antibiotic	Resistance µg/ml	References
pLOF/Km	Mobilizable transposon delivery plasmid carrying mini Tn10 with inducible transposase (oriVI of R6K, λ pir dependent)	Km <sup>r</sup>	<i>A. pp</i> 50 <i>E. coli</i> 50	Tascon et al., 1993
pGZRS-1	Endogenous 4.3-kb <i>A. pp</i> plasmid	Sm <sup>r</sup>	<i>A. pp</i> 100 <i>P. haem</i> 100 <i>E. coli</i> 50	West et al., 1995
pGZRS-18/19	Shuttle vector derived from pGZRS-1 with MCS, <i>lacZ</i>	Ap <sup>r</sup> (Tn3)	<i>A. pp</i> 25 <i>P. haem</i> 50 <i>E. coli</i> 50	West et al., 1995
pGZRS-38/39	Shuttle vector derived from pGZRS-1 with MCS, <i>lacZ</i>	Km <sup>r</sup> (Tn903)	<i>A. pp</i> 25 <i>P. haem</i> 50 <i>E. coli</i> 50	West et al., 1995
pJF224-NX/XN	Mobilizable broad host range expression shuttle vectors based on RSF1010, MCS, promoter cassette, phage T4 gene 32	Cm <sup>r</sup> (pS-a)	<i>A. pp</i> 1–2 <i>P. haem</i> 1–2 <i>E. coli</i> 25	Frey, 1992
pLS88	Endogenous <i>H. ducrei</i> plasmid used as shuttle vector with various Pasteurellaceae	Sm <sup>r</sup> Su <sup>r</sup> Km <sup>r</sup>	<i>A. pp</i> 50, 190, 30 <i>H. duc.</i> 50, 190, 30 <i>H. flu.</i> 50, 190, 30 <i>E. coli</i> 50, 190, 30	Willson et al., 1989
pMBK1	Mobilizable shuttle vector with <i>sacB</i> counterselection for introduction of marked deletions	Km <sup>r</sup> (Tn903)	<i>A. pp</i> 25 <i>E. coli</i> 50	Oswald et al., 1999
pEMOC2	Mobilizable shuttle vector with <i>sacB</i> counterselection	Cm <sup>r</sup>	<i>A. pp</i> 5	Baltes et al., 2003
pYP53	Shuttle vector with MCS based on <i>A. pp</i> plasmid pYG10	Cm <sup>r</sup> (pYG10)	<i>A. pp</i> 10 <i>E. coli</i> 10	Lalonde and O'Hanley, 1989
pVT736-1	Chimeric plasmid produced by ligating to an <i>A. a.</i> plasmid to pUC19	Ap <sup>r</sup> (puc19)	<i>A.a. E. coli</i>	Leblanc et al., 1993

Abbreviations: Km<sup>r</sup>, kanamycin resistance; Sm<sup>r</sup>, streptomycin resistance; Ap<sup>r</sup>, ampicillin resistance; Su<sup>r</sup>, sulfonamide resistance; Cm<sup>r</sup>, chloramphenicol resistance; MCS, multiple cloning site; *A.a.*, *Actinobacillus actinomycetemcomitans*, *A. pp.*: *Actinobacillus pleuropneumoniae*; *H. duc.*: *Haemophilus ducreyi*; *H. flu.*: *Haemophilus influenzae*; and *E. coli*: *Escherichia coli*.

four plasmids with molecular sizes 4–20 kDa. While other investigators have confirmed the presence of plasmids in (*A.*) *actinomycetemcomitans*, the occurrence was at a much lower frequency (LeBlanc and Lee, 1993). Of 39 isolates examined, only two harbored detectable plasmids. One strain contained two plasmids, 1.9 kb and ≥30 kb, and the other strain harbored a single 24-kb plasmid. It was the 1.9-kb plasmid, subsequently designated “pVT736-1,” that has been used as the core element in the construction of a number of shuttle plasmids.

The absence of a selectable phenotype in pVT736-1 initially made it unsuitable for use in a transformation system. This issue was remedied by ligation with *Escherichia coli* plasmids such as pUC19 and pGEM7Zf(-) resulting in chimeric plasmids that were able to replicate in several strains of (*A.*) *actinomycetemcomitans* following transformation by electroporation (Sreenivasan et al., 1991). A recombinant plasmid containing two antibiotic-resistant markers was subsequently developed (Sreenivasan et al., 1991; Sreenivasan and Fives-Taylor, 1994). This plasmid, pDL282, was constructed by insertion of a spectinomycin gene isolated from *Enterococcus faecalis* (LeBlanc et al., 1991) into a

unique restriction site of pVT736-1. The transformation efficiency of (*A.*) *actinomycetemcomitans* by pDL282 was similar to the parent plasmid. pDL282 has been used to construct a shuttle vector (pAES19) that is composed of the fragment of pDL282 containing the (*A.*) *actinomycetemcomitans* origin of replication and spectinomycin gene and from pUC19, the multiple cloning site, and the *lacZa* reporter gene (Nakano et al., 1996), which allows for colorimetric detection of plasmids with inserts and rapid sequencing using commercially available primers.

The naturally occurring plasmid (pYG10) from *Actinobacillus pleuropneumoniae*, which codes for chloramphenicol resistance, was shown to transform some strains of (*A.*) *actinomycetemcomitans* (Lalonde et al., 1989b; Sreenivasan et al., 1991). A derivative of this plasmid, pYG53, was modified to generate a shuttle vector capable of transforming both (*A.*) *actinomycetemcomitans* and *E. coli* (Lalonde and O'Hanley, 1989a; Brogan et al., 1996). In this construct, the Km<sup>R</sup> gene from pUC4K was ligated into pYG53 to create pYGK. A “low copy plasmid” (3–4

copies per cell in *E. coli*), pYGK is useful for working with genes that are either unstable or lethal in high-copy-number vectors (Brogan et al., 1994).

Electroporation is an efficient means for transferring genetic material into (*A. actinomycetemcomitans*) using conditions established for *A. pleuropneumoniae* and its naturally occurring plasmid, pYG10 (Sreenivasan et al., 1991; Brogan et al., 1994). Conjugation has also been reported as a means for transporting DNA into (*A. actinomycetemcomitans*). Using incompatibility group P (IncP) plasmid, RK2, as the mobilization plasmid, an *E. coli* harboring IncP was used as the donor in mating experiments with a rifampin-resistant, spontaneous mutant of (*A. actinomycetemcomitans*) as the recipient cell (Goncharoff et al., 1993). The successful transfer of the plasmid resulted in (*A. actinomycetemcomitans*) transconjugates that were both ampicillin- and rifampin-resistant. The frequency of Apr and Rif bacteria was obtained at a frequency of 0.3–0.5 transconjugates per donor cell.

Conjugation has also been used to introduce transposons into the (*A. actinomycetemcomitans*) genome. Insertional mutagenesis using a plasmid containing Tn916, a naturally occurring transposon of Gram-positive organisms, suggested that transformation of (*A. actinomycetemcomitans*) was possible (Sato et al., 1992). Kolodrubetz and Kraig (1994) then employed a suicide plasmid, pUT, to deliver a spectinomycin-resistant Tn5 derivative [mini-Tn5(Sp)] via conjugation from *E. coli* to (*A. actinomycetemcomitans*). Spectinomycin-resistant transconjugates were obtained at a frequency of about  $1 \times 10^{-7}$  per recipient cell, and Southern blot analysis showed that the transposon had inserted into the (*A. actinomycetemcomitans*) chromosome in a random manner in 32 different sites in the 33 exconjugants examined. Integration was stable, as demonstrated by the fact that all of the exconjugants remained spectinomycin-resistant after seven passages on nonselective media.

The genome of *Actinobacillus actinomycetemcomitans* strain HK1651 has been completely sequenced and annotated [NCBI website].

The strain sequenced was isolated from a patient with severe periodontal disease and contains the deletion in the leukotoxin promoter region that results in enhanced leukotoxin expression (Brogan et al., 1994), a characteristic that has been shown to be associated with severe localized aggressive periodontitis. The genome is 2,105,503 bp and is predicted to encode 2,345 open reading frames (ORFs). The G+C content of the genome (44.4 mol%) is similar to other previously sequenced members of the Pasteurellaceae family. In addition to containing six complete copies of the ribosomal RNA operons,

tRNAs representing each of the 20 amino acids have been identified. Annotation of the genome resulted in only 47.8% of the total predicted ORFs being assigned a function with the remaining 52.2% being comprised of conserved hypothetical proteins (32%) and hypothetical proteins (20.2%; A. Gillaspay and D. Dyer, personal communication).

The sequencing of *A. pleuropneumoniae* serovar 1, serovar 7 and two serovar 5 strains is well underway [NCBI website]. These whole genome sequences will be invaluable for physiology and pathogenesis of *A. pleuropneumoniae*, as well as establishing the relationships of these organisms to one another and to other members of the genus and the family Pasteurellaceae.

## Epidemiology

### (*Actinobacillus*) *actinomycetemcomitans*

This organism is associated with a form of periodontal disease known as localized juvenile (aggressive) periodontitis (LAP). A unique disease relative to other forms of periodontitis, LAP occurs in young people, a group that traditionally exhibits a low incidence of periodontal disease. Second, while most periodontal diseases appear to be polymicrobial infections, the subgingival microbiota in LAP typically demonstrates an unusually high association with a single bacterium, (*A. actinomycetemcomitans*). Zambon et al. (1983) reported that (*A. actinomycetemcomitans*) was cultured from 96.5% of LAP patients, but was recovered in only 15.2% of non-LAP patients (healthy individuals, adult periodontitis patients, and insulin-dependent diabetics). In late adolescence, bone loss decreases or arrests, leaving pathognomonic, arc-shaped bony defects around the permanent first molars and/or the incisors in otherwise healthy individuals (Baer, 1971). Another interesting feature of LAP is that the infection occurs in families. Children with LAP almost always have a parent and/or siblings with the disease. Two models could explain these clinical observations: 1) patients with LAP have a genetic defect that makes them susceptible to (*A. actinomycetemcomitans*) colonization or 2) there is vertical (but not horizontal) transmission of virulent strains of (*A. actinomycetemcomitans*).

Analysis of LAP families suggest that predisposition to the disease may be genetically regulated (Jorgenson et al., 1975; Melnick et al., 1976; Saxen, 1980; Saxen and Nevanlinna, 1984; Boughman et al., 1986; Beaty et al., 1987; Hart et al., 1992). Toward this end, a number of cellular defects have been reported in immune cells from patients with LAP including decreased



chemotaxis (Van Dyke et al., 1981; Sigusch et al., 2001), altered superoxide production (Hurttia et al., 1998; Biasi et al., 1999), decreased Ca<sup>2+</sup> mobilizations (Daniel et al., 1993), decreased phagocytosis (Sigusch et al., 1992), and defective formyl-methionyl-leucyl-phenylalanine (fMLP)-mediated chemotaxis (Perez et al., 1991; Sigusch et al., 1992), all of which suggest that LAP patients have increased susceptibility to (*A.*) *actinomycetemcomitans* infections. While most of these hypotheses are both attractive and plausible, they have not stood the test of independent verification.

On the other hand, there is increasing support for the fact that a unique serovar b clone, which is characterized by a 530-bp deletion (Brogan et al., 1994) in the promoter region of the RTX operon, can be isolated consistently from patients with active disease. Using polymerase chain reaction (PCR) techniques, this particular (*A.*) *actinomycetemcomitans* mutant has been demonstrated in LAP patients from diverse individual and geographic backgrounds such as African-Americans and Caucasians in the United States and Brazilians (Bueno et al., 1998; Haraszthy et al., 2000; Guthmiller et al., 2001), while others have isolated organisms with the same 530-bp deletion from African immigrants in European countries (Haubek et al., 1995; Haubek et al., 1996; Haubek et al., 1997) and demonstrated that this marker is endemically present in (*A.*) *actinomycetemcomitans* strains isolated from Moroccan children with LAP (Haubek et al., 2001). These data support a model whereby LAP is a vertically transmitted infection of a mutant (*A.*) *actinomycetemcomitans* to normal individuals.

### *Actinobacillus equuli*

Given the uncertainty associated with the classification of *A. equuli* and *A. equuli*-like organisms, discussion of the epidemiology of this organism is difficult. *Actinobacillus equuli* has long been associated with sporadic cases of disease in horses worldwide. From analysis of ribotyping and biotyping data, the population structure of *A. equuli* appears to be quite heterogeneous (Sternberg, 1998; Sternberg and Brändström, 1999). These findings are consistent with early studies by Kim et al. (1976) where more than 28 different heat-stable, antigenic groups were reported. On the basis of pulsed field gel electrophoresis (PFGE) analysis of 174 strains on 10 different farms in Sweden, no single strain of *A. equuli* seemed to predominate (Sternberg, 1998). The same strain was found in only 1 of 4 mother-foal pairs where *A. equuli* was isolated from both. Reminiscent of *Haemophilus parasuis* in swine, the author further showed that

multiple strains of *A. equuli* were present in the same herd and even in the same animal. In a subsequent study by Sternberg and Brändström (1999) where 112 isolates from clinical cases and healthy horses were evaluated by biochemical typing and ribotyping, there were no clear differences between strains from healthy and diseased animals. The inability to identify a “pathogenic phenotype” may have been due to some limitations of the sampling or reflect that all strains may be opportunistic pathogens. Given the demonstration of hemolytic *A. equuli*, which contains Aqx (a toxin to equine lymphocytes), it is tempting to speculate that these strains have greater pathogenic potential, but no experimental or epidemiological data support this notion at this time (Kuhnert et al., 2003).

### *Actinobacillus lignieresii*

This agent causes actinobacillosis, or wooden tongue, a chronic disease in cattle that can be confused with actinomycosis (due to *Actinomyces bovis*). The organism has a worldwide geographic distribution but clinical cases are generally sporadic (Campbell et al., 1975; Phillips, 1992). Subclinical cases may be overlooked so its distribution and importance may be underestimated. *Actinobacillus lignieresii* has been reported in the oral cavity and pharynx of healthy sheep and cattle, though in some early reports isolates identified as *A. lignieresii* were misclassified (Bisgaard et al., 1986). Similarly, *A. lignieresii* isolates reported in other hosts (e.g., rat, dog and duck) were likely other members of the family Pasteurellaceae. Recent studies of equine strains of *A. lignieresii* reveal that these organisms are genetically most closely related to Bisgard taxon 11 and *A. equuli* subsp. *haemolyticus*. Since no distinguishing phenotypic characteristics allow them to be separated from *A. lignieresii*, they have been designated “*Actinobacillus* genomospecies 1” (Christensen et al., 2002a). Six serovars of *A. lignieresii* have been described (one of which shares crossreactive epitopes with *A. pleuropneumoniae*; Lebrun et al., 1999), but the use of serotyping in epidemiological studies has not been reported. Such a study would be of interest as there is some evidence that particular strains of *A. lignieresii* are more virulent than others (Rycroft and Garside, 2000).

### *Actinobacillus pleuropneumoniae*

The causative agent of porcine pleuropneumonia, *A. pleuropneumoniae* has been reported in all countries where there is intensive swine rearing. Pleuropneumonia often occurs as an epizootic, but it is becoming endemic in many

countries. Two main biovars of *A. pleuropneumoniae* have been described on the basis of their requirement for NAD (Fodor et al., 1989). Biovar 1 strains require NAD, whereas biovar 2 strains can synthesize NAD in the presence of specific pyridine nucleotides or their precursors (Niven and Levesque, 1988). Serodiagnostic tests such as enzyme-linked immunosorbent assays (ELISAs) and the complement fixation test (CFT; Inzana and Fenwick, 2001; Enoe et al., 2002), as well as PCR-based tests (Schaller et al., 2001; Chiers et al., 2002) can be used to monitor the presence of *A. pleuropneumoniae* in swine herds. There are more than fifteen different serovars of *A. pleuropneumoniae*. Thirteen serovars (1–12, 15) of biovar 1 strains and 6 serovars (2, 4, 7, 9, 13, and 14) of biovar 2 have been described on the basis of surface polysaccharide antigens, primarily capsule (Nielsen et al., 1997; Blackall et al., 2002). The chemical structures of all of the capsular polysaccharides (CPS) and O side chains of the lipopolysaccharides are known for serovars K1 to 12 and O1 to 11, and a proposed comprehensive serotyping scheme was based on these two antigens (Beynon et al., 1992). Serovars 1 and 5 have been further differentiated into 1a and 1b, and 5a and 5b, on the basis of minor differences in the polysaccharide structures (Altman et al. 1992; Jolie et al., 1994). Although current data are not available from all countries, the most prevalent serovars in Asia and Australia are 1, 2 and 5. In Great Britain and Europe serovars 2 and 9 are found most often, whereas in Canada, the United States and Mexico, serovars 1 and 5 predominate. Although there are usually 1–3 predominant serovars, in some countries as many as 11 different serovars have been reported (Dubreuil et al., 2000). Recently, untypable strains that do not fall into a single serovar have appeared (B. Fenwick, personal communication). As well, strains that share antigen determinants of more than one serovar have been described (Gottschalk et al., 2000).

### *Actinobacillus suis*

Beginning in the early 1990s, the numbers of reports of severe *A. suis* outbreaks in Canada, the United States, and more recently Australia (MacInnes and Desrosiers, 1999; Taylor 1999b; Wilson and McOrist, 2000) have increased. In conventional herds, *A. suis* is a common commensal, and disease is sporadic. In specific pathogen-free herds, epidemics of severe *A. suis* disease can occur in pigs of all ages when the organism is first introduced. Once herd immunity has been established, however, the number of disease outbreaks decreases (Taylor, 1999b). Early studies of the population structure of *A. suis* revealed very little heterogeneity among *A.*

*suis* isolates cultured from healthy and diseased swine (Bada et al., 1996; Van Ostaaijen et al., 1997), but later studies by Slavic et al. (2000a) revealed that there are at least two O types of *A. suis*. These authors further demonstrated that O2 isolates were more likely to be associated with severe disease; however, they postulated that it was the CPS rather than the lipopolysaccharide (LPS) that may have been the more important determinant of pathogenesis (Slavic et al., 2000a; Slavic et al., 2000b).

## Disease

### *Actinobacillus actinomycetemcomitans*

This bacterium is a pioneer colonizer of the human upper aerodigestive tract, with colonization of the oral cavity occurring early in life when the native flora are being established and before tooth eruption has taken place (Alaluusua and Asikainen, 1988; Lamell et al., 2000; Morinushi et al., 2000; Tanner et al., 2002). To establish an ecological niche, the organism has developed strategies and amassed a diverse and rather impressive array of factors to fulfill its colonizer goal. These include 1) the ability to attach to extracellular matrix proteins (Mintz and Fives-Taylor, 1999) and epithelial cells (Meyer and Fives-Taylor, 1994; Mintz and Fives-Taylor, 1994a); 2) antibiotic resistance (Roe et al., 1995); 3) a bacteriocin (Hammond et al., 1987; Stevens et al., 1987); 4) bone resorption by either endotoxin (Kiley and Holt, 1980; Saglie et al., 1990) or surface-associated material (Saglie et al., 1990; Meghji et al., 1994); 5) a chemotactic inhibitor (Van Dyke et al., 1982; Meghji et al., 1994); 6) a collagenase (Robertson et al., 1982), 7) a cytolethal distending toxin (Shenker et al., 1982; Helgeland and Nordby, 1993); 8) Fc-binding proteins (Tolo and Helgeland, 1991; Mintz and Fives-Taylor, 1994b); 9) an RTX toxin (Baehni et al., 1981); and 10) the ability to invade either cells (Meyer and Fives-Taylor, 1993) or tissues (Christersson et al., 1987; Saglie et al., 1987). True to its pathfinder role, this is the only periodontal pathogen that is able to both survive with and kill human immune cells in co-culture (Baehni et al., 1979b).

A critical part of any virulence strategy begins with an ability to persist—the initial step that leads to colonization. The recent description of the *tad* (tight adherence) locus enables the (*A.*) *actinomycetemcomitans* to form biofilms that are difficult to disrupt. The 12–14 genes that make up the *tad* locus include *flp-1*, a pilin gene (Inoue et al., 1998; Kachlany et al., 2001b) and *tadABCDEF* (Kachlany et al., 2000), which are required for tight adherence and the associated

phenotypes of autoaggregation, rough colony morphology, and fibril production. Homologues of the *tad* loci have recently been implicated in human and animal diseases caused by *Pasteurella multocida* (Fuller et al., 2000a) and *Haemophilus ducreyi* (Nika et al., 2002). The observation that *tad*-like loci are found in other human pathogens, including *Yersinia* spp., *Bordetella pertussis*, *Pseudomonas aeruginosa*, *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis* lend support to the notion that colonization is important in microbial pathogenesis (Kachlany et al., 2001a; Planet et al., 2003).

The leukotoxin (Ltx) is a 116-kDa cytotoxic protein that specifically kills a subset of leukocytes including polymorphonuclear leukocytes and peripheral blood monocytes (Baehni et al., 1979a; Tsai et al., 1979). On the basis of sequence homology (Kolodrubetz et al., 1989; Lally et al., 1989a; Lally et al., 1989b; Kraig et al., 1990), the leukotoxin is a member of the RTX family of pore-forming bacterial toxins (Fig. 2). These toxins are synthesized by a diverse group of Gram-negative pathogens including other actinobacilli (see Table 3). All members of the RTX toxin family, including cytolytic toxins, metalloproteases and lipases, share a common gene organization and distinctive structural features. Although variations do exist, the generic RTX toxin operon consists of four genes that are designated *rtxC*, *A*, *B* and *D* in transcriptional order. RTX toxins require post-translational modification to become biologically active. This activation step is accomplished by fatty acid acylation of the structural *rtxA* gene product and requires the participation of the *rtxC* gene product and an acyl carrier protein (Issartel et al., 1991; Stanley et al., 1994; Stanley et al., 1998). Toxin produced in the absence of RtxC is biologically inactive. RTX toxins do not have canonical signal sequences and are transported from the cytoplasm to the cell surface by transport proteins encoded by the *rtxB* and *rtxD* genes. RTXA contains tandemly repeated nonapeptides that have the consensus sequence GGXGXDX (LIIIVWYIF)X (where G is glycine, D aspartic acid, L leucine, I isoleucine, V valine, W tryptophan, Y tyrosine, F phenylalanine, and X any

amino acid). The number of times this motif is repeated varies, ranging from six to 40 with individual toxins, but its presence is the sine qua non for RTX toxin family membership. RTXB proteins are members of the ATP-binding cassette (ABC) superfamily of transport proteins (Higgins, 1992), and RTXD proteins, which are unique to prokaryotes, belong to the membrane fusion protein family (Dinh et al., 1994).

Although extensive amino acid homology exists among the various RTX cytolysins, the cellular and species specificities remain unique for individual toxins (i.e., [*A.*] *actinomycetemcomitans* leukotoxin [LtxA] kills human monomyelocytes while a related toxin, *M. hemolytica* leukotoxin [LktA] kills bovine lymphoid cells). Studies with series of *ltxA/lktA* chimeric toxin genes showed that a 253-amino acid fragment (residues 688–941) that contains the GGXGXDX(LIIIVWYIF)X repeats is involved in target cell recognition.

The restricted host cell specificity of LtxA and LktA (Shewen and Wilkie, 1982; Strathdee and Lo, 1989) suggests that some, and perhaps all, RTX toxins exert their lethal effects by binding to cell-surface receptors. In addition to direct evidence, a general rule for bacterial toxins is that they possess domains for target cell recognition. Members of the colicin family bind to *E. coli* outer membrane proteins (Parker et al., 1990), *Bacillus thuringiensis*  $\delta$ -endotoxin attaches to the brush border of insect gut cells (Van Rie et al., 1990), recognition of a heparin-binding epidermal growth factor-like precursor is necessary for diphtheria toxin binding to target cells (London, 1992), and the family of AB<sub>5</sub> toxins (e.g., cholera toxin) bind to cell surface ganglioside lipids (Merritt and Hol, 1995). Believing that the unique specificity exhibited by RTX toxins is due to RTX recognition of a cell surface receptor led to the identification LFA-1 ( $\alpha$ L/ $\beta$ 2, CD11a/CD18) as a receptor for RTX toxins (Lally et al., 1997; Wang et al., 1998; Li et al., 1999; Ambagala et al., 1999). LFA-1 is a leukocyte-specific receptor that mediates cell-cell interactions in the immune system (Stewart and Hogg, 1996; Gahmberg, 1997).

In addition to the leukotoxin, all strains of *A. actinomycetemcomitans* also produce cytolethal distending toxins (Cdt) that interfere with the normal cell cycle progression of lymphocytes resulting in G<sub>2</sub> (the gap after S phase) arrest. The molecule responsible for this activity is *cdtB* gene product, a member of the family. The structural toxin gene, *cdtB*, is part of a three-gene operon in transcriptional order: *cdtA*, *cdtB*, and *cdtC*, which encode polypeptides with molecular masses of 24–35 kDa. While Cdts were first recognized by their ability to cause progressive cellular distension of G<sub>2</sub>-arrested epithelial cells

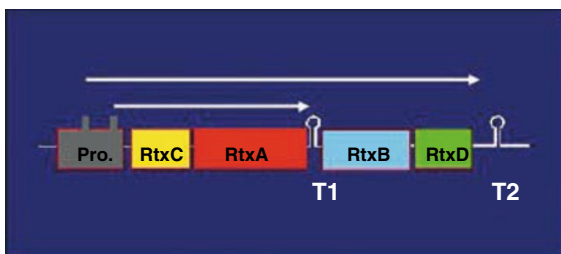


Fig. 2. Canonical RTX (repeats-in-toxin) operon.

that contain a tetraploid complement of DNA. Distension is much less evident in intoxicated lymphocytes. Cdts have also been identified in several enteric pathogens such as: pathogenic *Escherichia coli*, *Campylobacter jejuni*, *Shigella* species, *Haemophilus ducreyi* and now (*A. actinomycetemcomitans*) (Pickett and Whitehouse, 1999; Shenker et al., 2000; Shenker et al., 2001).

The combination of Ltx and Cdt provide *A. actinomycetemcomitans* with two very efficient protective mechanisms for dealing with either innate or adaptive immunity during the colonization process. Ltx provides the organism with an efficient “summary execution” capability as a way of dealing with LFA-1-bearing immune cells that include granulocytes, natural killer (NK) cells and macrophages. Cdt is able to nullify the adaptive arm of the immune system by blocking the Achilles heel of adaptive immunity—cell division. Arrest of the cell cycle blocks critical adaptive functions such as clonal expansion and cytokine production.

### *Actinobacillus equuli*

A common resident of the oral cavity of adult horses (Phillips, 1984; Phillips, 1992; Sternberg and Brändström, 1999), *A. equuli* can be transmitted from the mare to the foal via the oral, respiratory, or umbilical route at or following birth. It has also been suggested that the organism can be transmitted in utero. In young foals, *A. equuli* can cause an acute septicemia also known as “sleepy foal disease” (Rycroft et al., 1998; Quinn et al., 2002). In foals less than one month of age, *A. equuli* suppurative nephritis, arthritis, pneumonia, pleuritis or enteritis is frequently seen in association with septicemia. Virtually nothing is known about the virulence factors of *A. equuli*. Like *A. pleuropneumoniae* and *A. suis*, hemolytic strains of *A. equuli*, produce an RTX toxin (Berthoud et al., 2002; Kuhnert et al., 2003; Table 3). This toxin is encoded by the *aqxA* gene, which lies within a typical RTX cluster (*aqxCABD*). It has a predicted molecular mass of 110 kDa and has 9 characteristic glycine-rich nonapeptide repeats. The AqxA protein most closely resembles the LktA protein of *Mannheimia haemolytica*, whereas the B and D proteins have greater than 90% homology with the ApxIB and D proteins of *A. pleuropneumoniae*. Berthoud et al. (2002) found that the *aqxCABD* operon was present in all hemolytic strains of *A. equuli* (which they designated “*A. equuli*-like”), including two strains from rabbits. In contrast, these genes were absent in non-hemolytic *A. equuli*. In addition to its hemolytic activity, the AqxA toxin when expressed in *E. coli* had clear cytotoxic activity against horse lymphocytes but only weak cytotoxic activity

against porcine lymphocytes. It is tempting to speculate that the AqxA toxin may have a role in virulence, but this remains to be tested. It has been generally thought that all *A. equuli* have equal pathogenic potential. Consistent with this, Sternberg and Brändström (1999) reported that it was not possible to distinguish between clinical isolates and normal flora by either ribotyping or phenotyping, though unfortunately hemolytic activity was not evaluated.

### *Actinobacillus lignieresii*

This agent causes actinobacillosis or wooden tongue, a chronic disease in cattle that can be confused with actinomycosis (due to *Actinomyces bovis*), cancers and abscesses due to other organisms (Belschner, 1984). *Actinobacillus lignieresii* has a worldwide geographic distribution, but clinical cases are generally sporadic (Campbell et al., 1975; Quinn et al., 2002). Sub-clinical cases may be overlooked so its distribution and importance may be underestimated. Although there have been no systematic studies to identify the source of infection, it is a likely resident of either the rumen or oropharynx. The disease, which progresses very slowly, is normally limited to the soft tissues of the head and neck, particularly the tongue. Damage to mucous membranes predisposes animals to infection. Typically, multiple abscesses occur on the head and under the jaw and throat region. Small, hard, pus-filled ulcers may be present on the tongue. As the disease progresses, the throat region becomes involved, and the animal cannot retract its tongue. The lymphatic glands of the head and neck are often involved as well, and lesions may be present throughout the body. The lesions in the mouth can eventually lead to starvation, or pressure on the windpipe may lead to asphyxiation. Abscesses around the entrance to the rumen may cause chronic bloating and other digestive disturbances. Although animals mount a detectable humoral immune response to *A. lignieresii*, antibodies do not seem to be protective (Rycroft and Garside, 2000). Despite the fact that *A. lignieresii* and *A. pleuropneumoniae* share more than 70% DNA-DNA homology (Borr et al., 1991), the diseases caused by these organisms are very different. The virulence factors of *A. lignieresii* are unknown. *Actinobacillus lignieresii* carries homologues of the *A. pleuropneumoniae* *apxICA* genes, but promoter sequences are absent and the toxin is not expressed (Schaller et al., 2000).

### *Actinobacillus pleuropneumoniae*

All 15 serovars of *A. pleuropneumoniae* are capable of causing pleuropneumonia, although

some serovars are more virulent than others. Although the dogma was once that pigs that survived infection with one serovar were immune to infection with others, there is now evidence that convalescent pigs are only partially protected from challenge by heterologous serovars (Crujisen et al., 1995b). The differences in virulence among the serovars may be attributed, at least in part, to the production of different combinations of the Apx toxins with the most virulent serovars producing both ApxI and ApxII (Frey, 1995a; Table 3). Other factors, notably the amount of surface polysaccharide present, may also contribute to differences in virulence both between serovars and also between strains within the same serovar (Rosendal et al., 1985; Bandara et al., 2003). The pace of disease can range from peracute to chronic depending on strain, the immune status of the host, and the number of bacteria reaching the lung (Crujisen et al., 1995a). During peracute or acute disease, pigs may have a high fever, increased respiratory rate, coughing/sneezing, dyspnea, anorexia, ataxia, vomiting, diarrhea, and severe respiratory distress with cyanosis (Taylor, 1999a).

Early in the disease, neutrophil (PMN) infiltration is marked, while during later stages of the disease, macrophage infiltration is more apparent (Liggett et al., 1987). Severe necrotizing vasculitis leads to hemorrhage in the lung (Serebrin et al., 1991; Fig. 3). The organism can be found within alveolar and interlobular fluid and may spread within the lung via lymph vessels, but bacteremia is rare (Ajito et al., 1996). Animals that survive infection frequently retain focal necrotic sequestra, and chronically infected animals may also harbor *A. pleuropneumoniae* in tonsillar crypts (Sidibe et al., 1993; Fig. 4).

In contrast to *A. equuli*, *A. ligieresii* and *A. suis*, the pathogenesis of *A. pleuropneumoniae* infection has been extensively studied. Several adhesins have been proposed including capsule, fimbriae, and a 55-kDa outer membrane protein (OMP; Zhang et al., 2000; Labrie et al., 2002; Van Overbeke et al., 2002). As well, factors associated

with nutrient acquisition have also been described. *Actinobacillus pleuropneumoniae* has several mechanisms to overcome iron-limitation in the host. It is capable of utilizing heme compounds (including free heme, hemin, hematin and hemoglobin), as well as porcine transferrin, but not lactoferrin, for growth (Deneer and Potter, 1989; Niven et al., 1989; d'Silva et al., 1995). In addition, *A. pleuropneumoniae* can utilize exogenously supplied hydroxamate siderophores and may also liberate its own as yet uncharacterized siderophore (Diarra et al., 1996; Mikael et al., 2002). All serovars of *A. pleuropneumoniae* are capable of obtaining heme products via production of hemolysins (Frey et al., 1993), and all serovars secrete a high molecular mass protease complex ( $\geq 200$  kDa), which has weak activity against porcine hemoglobin in vitro (Negrete-Abascal et al., 1998; Garcia-Cuellar et al., 2000).

Several high-affinity receptors that bind the various iron chelates at the cell surface of *A. pleuropneumoniae* have been described. These include the TbpA (Tbp1, TfbB) and TbpB (Tbp2, TfbA), proteins for high-affinity binding of porcine transferrin (Gerlach et al., 1992; Gonzalez et al., 1995; Fuller et al., 1998) and FhuA, a receptor for ferric hydroxamate siderophores (Mikael et al., 2002). In a number of Gram-negative bacteria, it has been established that release of the strongly bound iron-complexes from high-affinity outer membrane (OM) receptors and subsequent transport across the OM requires energy that is provided by a TonB-ExbB-ExbD protein complex (Braun and Killmann, 1999; Braun, 2003). Iron-regulated *exbB* and *exbD* genes are present upstream of the *tbpA* and *B* genes, and further the role of the transferrin binding proteins in virulence has been demonstrated (Baltes et al., 2001). By contrast, there are no *exbB* or *exbD* genes linked to *fhuA*, and FhuA does not appear to be required for virulence (Baltes et al., 2003).

As with other Gram-negative bacteria, a periplasmic ABC transport system is thought to mediate passage of iron-carrier complexes to the

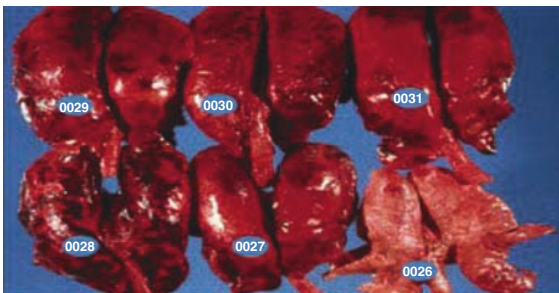


Fig. 3. Lungs from pigs with acute pleuropneumonia. Note that the lungs in the bottom right corner are almost normal.

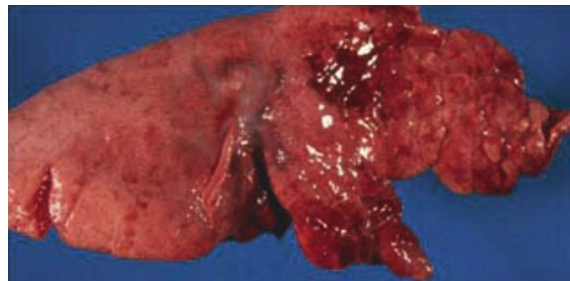


Fig. 4. Lung lobe from pig with chronic pleuropneumonia. Note the extensive fibrin deposits.

cytoplasm (Genco and White Dixon, 2001). In *A. pleuropneumoniae*, two ABC-transport systems have been reported to be involved in transfer of iron across the cytoplasmic membrane (CM). The FhuBCD system is specific for ferric hydroxamate (Mikael et al., 2002). A second periplasmic binding-protein-dependent iron transport system encoded by the *afuABC* genes has been described, but the role of these genes in iron uptake remains to be established (Chin et al., 1996; Braun and Killmann, 1999).

Several other ABC-transport systems have also been described that appear to be involved in the pathogenesis of *A. pleuropneumoniae*, or at least in its survival in the host. Recent signature-tagged mutagenesis studies of *A. pleuropneumoniae* have revealed ABC-transport homologues associated with the uptake of polyamines and sugars. Trace metals are required for survival within pig lungs, but once again, further study is needed to determine their precise role in pathogenesis (Fuller et al., 2000b; Sheehan et al., 2003). As well, the high affinity nickel uptake ABC-transport operon, *cbiKLMQO*, appears to have a role in pathogenesis (Bossé and MacInnes, 2000). The contribution of nickel to virulence of *A. pleuropneumoniae* is likely via its role in urease activity since a mutation within the urease operon leads to a similar level of attenuation. It has been postulated that urease activity could play a role in nutrient acquisition by producing ammonia, a preferred nitrogen source, and data also suggest that urease activity may cause impairment of the local immune response and slightly improve persistence (Baltes et al., 2001; Bossé et al., 2001).

Both macrophages and PMNs can phagocytose *A. pleuropneumoniae* in the presence of convalescent pig serum (Crujisen et al., 1992; Cullen and Rycroft, 1994), but only PMNs can effectively kill *A. pleuropneumoniae*. *Actinobacillus pleuropneumoniae* produces several factors that may contribute to its ability to survive within macrophages. High-molecular-weight carbohydrates present in the capsule and LPS may participate in scavenging of free toxic oxygen radicals (Bilinski et al., 1991; Ward et al., 1998; Rioux et al., 2000). In addition, SodC may have a role in dismutation of superoxide radicals in phagocytic cells (Langford et al., 1996), but isogenic *sodC* mutants are still capable of causing acute pleuropneumonia in experimental infections (Sheehan et al., 2000). Genes encoding two stress response proteins, DnaK and Trigger Factor, are also reported to be essential for survival of *A. pleuropneumoniae* within the porcine respiratory tract and could be important for survival within macrophages (Fuller et al., 2000b). Ammonia produced as the result of urea hydrolysis may also play a role in intracellular

survival (Bossé and MacInnes, 2000) since ammonia inhibits phagosome-lysosome fusion, as well as elevates intra-lysosomal pH in macrophages, resulting in depression of acid hydrolase activity (Gordon et al., 1980).

*Actinobacillus pleuropneumoniae* is resistant to the bactericidal effects of normal and immune serum and to complement-mediated opsonophagocytosis (Rycroft and Cullen, 1990; Thwaites and Kadis, 1993; Ward and Inzana, 1994). The main factors contributing to serum resistance by this bacterium are capsular polysaccharide (CPS) and/or LPS (Inzana et al., 1988; Rioux et al., 2000). Strains with thick adherent polysaccharide layers do not prevent activation of complement or binding of C3, but binding of anti-polysaccharide antibodies occurs at a distance from the cell membrane, so that deposition of C9, a component of the membrane attack complex, is limited (Ward and Inzana, 1994). Early studies with an undefined capsule-deficient serovar 1 mutant indicated that capsule was important in survival in the host. Similarly, a capsule-deficient serovar 5 strain with a defined mutation was effectively killed in the presence of normal and immune serum, whereas the encapsulated parent was not (Ward et al., 1998). In contrast, a serovar 1 capsular mutant created by transposon mutagenesis appeared to be fully resistant to killing by normal pig serum (Rioux et al., 2000). In a separate study, expression of LPS O-side chains was shown to be responsible for serum resistance in this serovar 1 strain (Paradis et al., 1999).

Although many of the pathological consequences of *A. pleuropneumoniae* infection have been attributed to LPS, extremely large doses of purified LPS (40–100 mg) are required to induce lesions similar to those found in naturally infected pigs (Fenwick et al., 1986). Furthermore, pigs infected with a mutant of a serovar 1 strain of *A. pleuropneumoniae* lacking ApxI and ApxII, but with normal LPS, did not develop clinical disease or significant lung lesions, suggesting that the contribution of LPS to lesion development may be minimal in the absence of Apx toxins (Tascon et al., 1994).

The major factors involved in impairment of phagocytic function of both macrophages and PMNs are three RTX-toxins (ApxI, ApxII and ApxIII), which are produced in various combinations by the different serovars of *A. pleuropneumoniae* (Frey, 1993; Table 3). At sublytic doses, these toxins impair macrophage chemotactic and phagocytic function but stimulate macrophage and PMN oxidative metabolism (Dom et al., 1992a; Dom et al., 1992b; Tarigan et al., 1994). At high concentrations, ApxI and ApxIII are highly toxic, whereas ApxII is moderately toxic for alveolar macrophages and PMNs

(Kamp et al., 1991; Rycroft et al., 1991; Frey, 1993). The Apx toxins are also associated with direct damage to endothelial cells (Serebrin et al., 1991). The critical role of Apx toxins in development of clinical disease and tissue damage has been confirmed using recombinant toxins (Kamp et al., 1997). Endobronchial inoculation of pigs with either rApxI or rApxIII results in severe clinical disease and lesions indistinguishable from those seen in acutely infected animals, whereas inoculation with rApxII results in few or no clinical signs of disease and only mild lung lesions. Although ApxII appears to contribute only minimally to lesion formation, serovar 7 strains, which produce only ApxII, are capable of causing severe disease with typical lung lesions, suggesting other factors are involved (Frey, 1993; Kamp et al., 1997). Similarly, damage to cultured alveolar macrophages by an ApxII and ApxIII-deficient mutant of *A. pleuropneumoniae* serovar 2 suggests that pathogenesis is complex (Cullen and Rycroft, 1994). Recently, a fourth RTX toxin (ApxIV) was reported to be produced by all serovars of *A. pleuropneumoniae* (Schaller et al., 1999). This toxin is antigenically distinct from the other Apx toxins and is only produced in vivo. *Escherichia coli*-expressing cloned ApxIV, along with an associated upstream ORF, has weak hemolytic and co-hemolytic (CAMP) activities (Schaller et al., 1999). The contribution of this RTX toxin to pathogenesis of *A. pleuropneumoniae* remains to be elucidated.

Apart from producing Apx toxins and LPS, *A. pleuropneumoniae* also secretes proteases into the culture medium that may contribute to the pathogenesis of infection (Negrete-Abascal et al., 1998; Garcia-Cuellar, 2000). Recently, a 24-kDa zinc metalloprotease that is present in all *A. pleuropneumoniae* serovars was cloned and expressed in *E. coli*. The oligomeric, but not the monomeric, form of the recombinant polypeptide cleaved azocoll, gelatin, and actin in vitro. This protease is probably different from the >200-kDa metalloprotease complex described by Negrete-Abascal et al. (1998) that also degrades gelatin, since antibodies against this protein fail to recognize the recombinant 24-kDa protease. Although the presence in convalescent antiserum of antibodies specific to the proteases indicates that they are produced in vivo (Negrete-Abascal et al., 1998; Garcia-Cuellar et al., 2000), their possible contribution to development of pathology has yet to be investigated.

### *Actinobacillus suis*

In conventionally reared swine, *A. suis* is a common commensal organism of the upper respiratory tract and an opportunistic pathogen,

especially in very young animals. In high-health status herds, *A. suis* may not be present and epidemics of severe *A. suis* disease can occur in animals of all ages if the organism is introduced. Once herd immunity has been established, however, the number of disease outbreaks decreases (Taylor, 1999b). In neonates and suckling pigs, *A. suis* can cause an acute and rapidly fatal septicemia where death occurs within 15 h. Affected animals may show signs of cyanosis, petechial hemorrhage, fever, respiratory distress, neurological disturbances, and arthritis. In slightly older animals, the disease tends to be less severe and may be characterized by fever, anorexia, and persistent cough. Although mortality is much lower, these animals are often "poor doers" (i.e., consume large amounts of feed but still appear thin). Erythematous skin lesions, fever, and anorexia/inappetence, abortion, metritis, and meningitis have also been reported in sows. Once in the bloodstream, *A. suis* can form microcolonies on vessel walls that lead to regions of hemorrhage and necrosis. Gross lesions are usually seen in the lungs, kidney, heart, spleen, intestines and skin. The lungs may also be filled with a serous or serofibrinous exudate and superficially look like lungs of animals with pleuropneumonia. Occasionally, animals are seen with an acute necrotizing myocarditis that is reminiscent of mulberry heart disease (MacInnes and Desrosiers, 1999).

In contrast to *Actinobacillus pleuropneumoniae*, relatively little is known about the pathogenesis of *Actinobacillus suis*, in part because there have been no good experimental challenge systems available (Slavic, 2000b). Despite many superficial similarities, *A. pleuropneumoniae* and *A. suis* share less than 50% DNA-DNA homology (Borr et al., 1991). They cause distinct diseases and presumably employ different virulence factors. Although *A. suis* is generally thought to be less pathogenic, it seems better able to invade the bloodstream, and unlike *A. pleuropneumoniae*, it can be isolated from the alimentary tract. To date, no attachment factors have been described for *A. suis*. As with *A. pleuropneumoniae*, fimbriae or surface polysaccharides or OMPs could play a role in attachment, but this remains to be tested. A number of virulence factors associated with nutrient acquisition are also present in *A. suis*. For example, *A. suis* is strongly urease positive and can bind porcine (but not human or bovine) transferrin using TbpA and TbpB. These proteins are virtually identical to those in *A. pleuropneumoniae* (Bahrami et al., 2003). Like *A. pleuropneumoniae*, the *exbB* and *exbD* genes are linked to the *tbpA* and B, but a *tonB* homologue is also present upstream. Except in the case of direct entry in the bloodstream (e.g., via an umbilical lesion), the mecha-

nism of invasion is another area of *A. suis* pathogenesis that is not understood. Once in the bloodstream, capsule and LPS undoubtedly play a role in the survival of the organism. Regardless of their O and K types, all *A. suis* isolates appear to be serum resistant and grow better in the presence of fresh serum than in serum that has been complement depleted (Slavic et al., 2000b). The RTX toxins, ApxI and ApxII, which are virtually identical to those present in *A. pleuropneumoniae*, also likely play a role in the pathogenesis of *A. suis*, but it appears that the genes are expressed at a lower level or that the activated toxin is transported outside the cell less efficiently (Van Ostaaijen et al., 1997). In contrast to *A. pleuropneumoniae*, *A. suis* lacks the *apxIV* genes (Schaller et al., 1999).

## Applications

A large number of *A. pleuropneumoniae* vaccines have been developed. Most of the early vaccines were whole cell preparations of multiple serovars. Later, purified Apx toxins and iron-regulated proteins were added to some preparations (Goethe et al., 2000). Particularly when compared with protection through natural infection, protection with these products tends to be incomplete (Furesz et al., 1997). A defined capsular mutant has been licensed for use in the United States (Ward et al., 1998), and several attenuated strains that secrete active or detoxified Apx toxins have been described (Prideaux et al., 1999; Fuller et al., 2000c; Garside et al., 2002), but their use may be limited by regulatory considerations. Genetically inactivated strains (bacterial ghosts) created via expression of a cloned phage lysis gene have also been explored as *A. pleuropneumoniae* vaccines (Huter et al., 2000).

At present, there are no commercial vaccines for *A. suis*; however, autogenous bacterins are thought to be useful (Lapointe et al., 2001). In theory, *A. pleuropneumoniae* vaccines, especially those with Apx toxins, might provide some cross-protection, but this remains to be proven.

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## The Genus *Francisella*

FRANCIS NANO AND KAREN ELKINS

### Introduction

*Francisella tularensis* is best known as the zoonotic, highly infectious etiologic agent of the human disease, tularemia. There are three recognized species in the genus *Francisella*: *F. tularensis*, *F. novicida*, and *F. philomiragia*. All three species can cause human infections, although *F. novicida* and *F. philomiragia* rarely do. Disease caused by *F. tularensis* can be mild but it usually is acute, severe and febrile; infection with a common North American strain can be life threatening.

### Phylogeny

Analysis of the 16S rRNA of *Francisella* species places them in the  $\gamma$ -subclass of the Proteobacteria (Forsman, 1994). All of the *Francisella* are closely related (98.5 to 99.9% sequence identity in the 16S rDNA sequence) and form a group together with *Wobachia persica* (97.85 identity) (Forsman, 1994). Recently, two groups discovered endosymbionts of the tick species, *Ornithodoros moubata* (Noda, 1997) and *Dermacantor andersoni* (Niebylski, 1997) that could be placed in the *Francisella* group by virtue of their 16S rDNA sequence similarity. The bacterium associated with *D. andersoni* could grow in Vero cells and infect rodents experimentally, although no tick-to-mammal transmission was observed. It is reasonable to assume that *W. persica* is misclassified as a *Wobachia* species and eventually will be recognized as *Francisella* species along with the tick endosymbionts.

### Taxonomy

Classically, *Francisella* spp. have been classified primarily by their virulence phenotypes. The most virulent form is often called *F. tularensis* type A but is also referred to as *F. tularensis* var. *tularensis*. This strain is found only in North America and is the usual cause of tularemia in

humans. The type B form of *F. tularensis*, sometimes referred to as var. *palaeartic*, is found throughout the Northern Hemisphere. This strain is less virulent than the type A strain. The type A form has citrulline ureidase and ferments glycerol, whereas the type B strain does not. *F. novicida* can be differentiated from the *F. tularensis* strains by its low virulence in rats, its ability to ferment sucrose, its rapid growth and its less fastidious requirements on agar media (Hollis, 1989; Owen, 1964).

### Habitat

*F. tularensis* and *F. novicida* probably should be considered obligate pathogens. Their fastidious growth requirements make growth outside of hosts unlikely. Many researchers have long considered the natural reservoir for *F. tularensis* type A to be the rabbit. In Europe and Asia, *F. tularensis* type B is most often associated with hares and small rodents. However, these associations are based on the observed transmission of *F. tularensis* to humans, and there may be other, unobserved, carriers of tularemia. One thing is clear: arthropods are simply a mechanical vector for transmission, and there is no transovarian passage of classical *F. tularensis* strains.

### Isolation

Because of the highly infectious nature of *F. tularensis*, its isolation usually is attempted only by laboratories equipped to handle level III pathogens. Typically, tissue or blood suspected of being infected is inoculated onto cysteine heart agar (Difco) supplemented with 5% (v/v) horse or rabbit blood or 1% (w/v) hemoglobin. The agar plates are incubated at 37°C, and gray colonies appear in 3–4 days. An environment of 5% CO<sub>2</sub> may help the growth of *F. tularensis* but is not essential. A more reliable method for isolating *F. tularensis* is to inoculate the sample into a mouse.

If *F. tularensis* is present, it will cause severe disease in the mouse and can be easily isolated from the liver or spleen.

It is thought that *F. tularensis* requires cysteine for growth on agar plates, and that *F. novicida* does not. This notion has not been systematically tested, and *F. tularensis* clinical isolates that lack a requirement for cysteine have been found (Bernard, 1994; Clarridge, 1995).

## Identification

The primary clue to the identification of *F. tularensis* is its association with a severe, flu-like illness contracted from an arthropod vector or contact with wild animals. Upon isolation, *F. tularensis* can be identified as a Gram-negative, small ( $0.2 \times 0.2\text{--}0.7$  (μm)) coccobacillus, with fastidious growth requirements. *F. novicida* and *F. philomiragia* cells appear slightly larger than those of *F. tularensis*. Most often, *F. tularensis* is positively identified by the reaction of specific antiserum. Traditionally, clinical laboratories have used hyperimmune, polyclonal antiserum for fluorescent microscopy or agglutination. Monoclonal antibodies that react with the O-antigen of the *F. tularensis* lipopolysaccharide (LPS) are equally useful for these techniques. Sufficient DNA sequence information exists to use polymerase chain reaction (PCR) or hybridization with oligonucleotides to identify *F. tularensis*, *F. novicida*, and *F. philomiragia* (Forsman, 1990; Fulop, 1996; Long, 1993). Members of the genus also can be identified by their unusual lipid composition (Jantzen, 1979; Hollis, 1989), which is especially important for the identification of *F. novicida* and *F. philomiragia* inasmuch as reagents to help in their identification are not commercially available. All of the *Francisella* species have  $C_{18}$  to  $C_{26}$ , saturated and monounsaturated fatty acids, large amounts of  $C_{10:0}$ ,  $C_{14:0}$ , and  $C_{16:0}$  saturated fatty acids, and two hydroxy acids, 3-OH  $C_{16:0}$  and 3-OH  $C_{18:0}$ .

## Preservation

Although some strains of *Francisella* can be preserved at  $-80^{\circ}\text{C}$  in 10% glycerol, other strains are lysed under these conditions and lose much of their viability. A more reliable preservation approach is to freeze or lyophilize *Francisella* strains in the presence of a 1–2% (w/v) protein-rich solution, such as gelatin or fetal bovine serum, or with an equal volume of sterile skim milk. Preserving *Francisella* strains also can be done for several months at  $-20^{\circ}\text{C}$ .

## Genetics

### Transformation and Genetic Manipulations in *Francisella*

All of the published genetic studies of *Francisella* have been done with the live vaccine strain (LVS) of *F. tularensis* or with *F. novicida*. Researchers have avoided genetic studies of the virulent strains to avoid infection of laboratory personnel and to avoid creating an antibiotic-resistant virulent strain. DNA can be introduced into *F. tularensis* LVS and into *F. novicida* via electroporation or chemical transformation (Anthony, 1991b; Tyeryar, 1970a; Tyeryar, 1970b). When *F. tularensis* is electroporated with cloned *F. tularensis* DNA interrupted with an antibiotic marker, the marker is rescued by the homologous recombination of the cloned DNA into the chromosome. The highest frequencies that have been observed are about 6,000 transformants per microgram of DNA. An *E. coli*-*Francisella* shuttle plasmid was constructed by joining parts of a plasmid found in an *F. novicida* strain with the origin of replication of pACYC184 (Norqvist, 1996; Pavlov, 1996). The plasmid can be introduced into *F. tularensis* LVS using a cryotransformation procedure (Mokreivich, 1994).

*F. novicida* can be transformed (Tyeryar, 1970a, 1970b) after growth in a defined medium (Chamberlain, 1965) and treatment with calcium. A stretch of *Francisella* DNA containing an antibiotic-resistance marker is rapidly integrated into the *F. novicida* chromosome (Anthony, 1991b). Transformation frequencies of approximately 100,000 per microgram of DNA were observed for circular DNA, and transformation frequencies of greater than one million per microgram of DNA were observed for linear DNA. Typically, linear DNA transforms at a much higher frequency. Broad host range plasmids carrying a *Francisella* insert can be found to replicate autonomously in *F. novicida* (Anthony, 1991b; Pomerantsev, 1991). With the exception of plasmid Sa, no DNA has been introduced and maintained by *F. tularensis* or *F. novicida* unless it contained some *Francisella* sequences. Attempts to introduce broad host range plasmids or transposons into *F. tularensis* or *F. novicida* by electroporation or chemical transformation have failed unless *Francisella* DNA cloned into the plasmid or physically linked to the transposon. The data suggest that transforming DNA needs homologous *Francisella* sequences for uptake or maintenance of the DNA. Despite the difficulties in defining the mechanism of DNA uptake, the high frequency transformation of *F. novicida* has allowed the development of methods for random transposon

mutagenesis, merodiploid formation, and gene fusions (Baron, 1998; Baron, 1999a; Mduli, 1994).

All *Francisella* strains tested express a naturally occurring  $\beta$ -lactamase. Most type B strains of *F. tularensis* are resistant to erythromycin, and most type A strains and *F. novicida* are sensitive to erythromycin. All strains are apparently sensitive to chloramphenicol, tetracycline and aminoglycosides, and these sensitivities allow for the use of antibiotic-resistance markers. Both *F. novicida* and *F. tularensis* LVS have been mutagenized with a mini-Tn10 carrying the kanamycin resistance marker from Tn903. To date, the most useful transposon for mutagenesis of *F. novicida* is a Tn1721-based minitransposon, TnMax2, which carries an erythromycin resistance cassette (Haas, 1993). A minicassette and a minitransposon were derived from TnMax2, which allow chloramphenicol acetyltransferase transcriptional fusions to occur in *F. novicida* (Baron, 1999a). All of these experiments relied on mutagenesis of *Francisella* DNA in *E. coli* and subsequent integration into the *Francisella* genome.

### Identified Genetic Loci and Sequenced Genes

A handful of loci have been molecularly cloned from *Francisella* and cognate insertion mutants created. An *F. novicida* *recA* mutant has been made, and it is unable to integrate DNA into its chromosome (Berg, 1992). A gene encoding an outer membrane protein, FopA, has been inactivated (Anthony, 1991b). An *F. novicida* *minD* insertion mutant was constructed and found to be sensitive to complement and oxidative radicals (Anthony, 1994). When this mutant was introduced into macrophages, viable numbers of the mutant dropped sharply, presumably from the oxidative burst, and then grew normally. A locus that appears to be needed for detergent and complement resistance, *valAB*, has been identified in *F. novicida* (Mduli, 1994). Using an allele that encodes a temperature sensitive form of ValA, researchers demonstrated that this locus is needed for the assembly of LPS (McDonald, 1997). Cumulative evidence from different publications suggests that ValA and its homologues are ABC transporters responsible for transport of lipid A across the inner membrane. The Val B molecule (called LpxK or OrfE in *E. coli*) is a lipid A 4'-kinase (Garret, 1997). All *Francisella* strains tested have a respiratory burst-inhibiting acid phosphatase, AcpA (Reilly, 1996). Inactivation of the encoding gene does not lead to loss of virulence in *F. novicida*. There is a second acid phosphatase in *F. novicida* that is not found in other *F. tularensis* strains (Baron, 1999b).

An apparent global regulator operon, *mglAB*, is needed for *F. novicida* intracellular growth and virulence (Baron, 1998). To date, this is the only locus in *Francisella* that has been definitively associated with a virulence phenotype. A missense mutation in *mglA* or insertional inactivation of *mglA* or *mglB* results in complete loss of the ability of *F. novicida* to grow inside macrophages. Complementation of the mutated *mglAB* with a wild-type form restores the ability of *F. novicida* to grow in macrophages. Inactivation of *mglAB* leads to loss of expression of several proteins, including a secreted 70-kDa protein.

One study examined the change in protein expression upon infection of macrophages by *F. tularensis* (Golovliov, 1997). A few proteins showed a small increase in expression within 24 hours after infection. Most notable was a 23-kDa protein. This protein also is induced by exposure of *F. tularensis* to hydrogen peroxide. The protein is localized in the cytoplasm, suggesting that it is a stress-induced protein that may be involved in gene regulation. It has no significant similarity to proteins of known function.

The DNA sequences of a handful of other genes have been described, but their role in *Francisella* virulence has not been studied. A combination of groups has embarked upon sequencing the entire genome of *F. tularensis*, Schu4, a highly virulent isolate. As of summer 1999, approximately 9 million bases had been sequenced, which can be accessed from the *Francisella* genome (<http://www.medmicro.mds.qmw.ac.uk/ft/>). Because the *Francisella* LVS genome appears to be about 2.5 Mb, it is likely that most of the *Francisella* genome is already sequenced. If the Schu4 strain genome is of similar size to that of the LVS strain, then one can expect that a great deal of information will be available soon about the *F. tularensis* genome.

### Variation in *F. tularensis*

In the 1950s Eigelsbach and colleagues identified spontaneous colony variants of *F. tularensis* that differed in appearance, immunogenicity, and virulence (Eigelsbach, 1951). Variants that were low in immunogenicity and virulence appeared "gray" when viewed through a dissecting microscope using oblique lighting; the wild-type colonies appeared as light blue. The difference is used to evaluate preparations of the tularemia vaccine. Vaccine preparations that contain significant numbers of "gray" variants are poorly immunogenic and not appropriate for human vaccination.

Recent work suggests that the basis for the change in colony morphology can be attributed to a shift in the amount and type of O-antigen produced (Cowley, 1996). The "gray" variants

appear translucent on blood agar plate and produce less of the usual *F. tularensis* O-antigen. Accompanying the lower expression of the *F. tularensis* O-antigen is a newly expressed O-antigen, of the type normally found on *F. novicida*. The change in O-antigen is coincident with a change in the ability of the LPS to induce nitric oxide production and for *F. tularensis* to grow in rat macrophages. These phenotypic changes are reversible and occur in one step. The genetic basis for these changes is not known but may become evident when the *Francisella* genome project is completed.

## Epidemiology

Virtually all available epidemiological information concerns *F. tularensis*. *F. novicida* (“new killer”) has been isolated only in the United States, once from water collected near dead muskrats in Utah (Larson, 1955) and twice as a human pathogen from patients with a tularemia-like illness (Hollis, 1989). The other species, *F. philomiragia*, originally was isolated from muskrats and water during an attempt to reisolate *F. novicida* (Jensen, 1969); this bacterium, named because of frequent mirages seen in the area, has been associated with an acute febrile disease in near-drowning victims and in patients with chronic granulomatous disease (Wenger, 1989; Sicherer, 1997). To date, 13/14 *F. philomiragia* isolates were from the United States, the other was from Switzerland (Wenger, 1989).

*F. tularensis* originally was isolated by McCoy and Chapin (McCoy, 1912) following outbreaks of a plague-like illness among ground squirrels in Tulare County, California. The agent responsible for this disease in rodents was also responsible for human illnesses, variously called lemming fever, deer-fly fever, rabbit fever, and tick fever in the United States. A similar clinical syndrome recognized among people who ate rabbit meat in Japan was called Ohara’s disease (Francis, 1925; Francis, 1928; Ohara, 1954). As implied by several of these early names, *F. tularensis* is carried by both arthropod vectors (such as ticks and deer flies) and by many species of vertebrates. Specifically, *F. tularensis* has been isolated from over 50 arthropod species, including a wide variety of ticks that live in association with wildlife and domestic animals. Further, *F. tularensis* has been isolated from over 100 types of wildlife; the most important animal reservoirs include rabbits, hares, mice, rats, muskrats, beavers, and voles. Tularemia has been diagnosed in, and acquired from, domestic cats as well (Capellan, 1993). Those most often afflicted with tularemia include hunters, hikers, and others who are exposed to wild animals and their arthropod par-

asites. Understanding the distribution of *Francisella* in nature is, therefore, key to appreciating its biology as well as its potential as a human clinical problem.

*F. tularensis* causes disease in virtually all its animal hosts, although incubation times and severity of symptoms vary widely. Not surprisingly, infected wild animals that are ill may come in contact with humans, with the end result being transmission of disease from animal to human. Person-to-person transmission occurs rarely, if ever. However, well-investigated outbreaks of pneumonic tularemia in Martha’s Vineyard (Teutsch, 1979) and in Sweden (Dahlstrand, 1971) also demonstrate that tularemia occasionally can be transmitted by aerosol. In Martha’s Vineyard a cluster of cases occurred in vacationers who were all exposed to the same indoor fire, while in Sweden a large outbreak occurred among farmers who inhaled dust from infected hay.

There are two seasonal peaks of *Francisella* infections. Disease diagnosed in the spring or early summer often is associated with tick or mosquito bites, when human outdoor activity and insect exposure are at their highest. Outbreaks of disease in the winter often are most associated with hunting.

Since its original description in the United States, *F. tularensis* has been isolated regularly in Canada, several states of the former Soviet Union, Sweden, Finland, Norway, Italy, Austria, Czechoslovakia, Hungary, and Japan. Virtually all isolates have been found in northern latitudes between 30 and 71°. Over 22,000 cases of tularemia were diagnosed in the United States between 1927 and 1948, with a case fatality rate of about 8% (Sanford, 1983); in the last three decades, however, only 100–300 cases per year are reported in the United States, with dramatic drops in case fatalities since the introduction of antibiotic treatment. The reasons for the decline in the overall number of cases in the United States since the 1950s are not clear but may be related to changes in housing patterns and in interaction with the relevant wildlife, either in the recreational or peri-residential environment.

Nonetheless, several well documented recent outbreaks of tularemia in the United States serve to illustrate the epidemiology of the disease in North America (Young, 1969; Brachman, 1969). Tularemia has been a reportable disease in the United States for several decades but had never occurred in Vermont until the spring of 1968. A cluster of tularemia cases in residents who had influenza-like symptoms with hand sores and lymphadenopathy was recognized in Vermont in early 1968. All cases had handled muskrats trapped from three streams in Addison County. Once the outbreak was appreciated, thorough

epidemiological investigation revealed a somewhat unexpected clinical picture. Thirty-nine symptomatic cases were identified, but the distribution between clinically mild, moderate, and severe disease was approximately even (11, 13, and 14 cases, respectively). This contrasts with the usual concept of tularemia as a severe systemic illness in all persons infected with the same virulent strain (which is assumed in this localized outbreak), suggesting large differences in host susceptibility to disease. In addition, serological screening of area residents who had contact with muskrats resulted in detection of eight apparently asymptomatic cases. *F. tularensis* subsequently was cultured from about 5% of captured muskrats in the area (but not from rabbits or from the ectoparasites combed from the muskrats), as well as from mud and water in the streams in question. Studies of the isolates from both dead muskrats and mud indicated that these were type B bacteria (Hornick, 1969), which are generally considered to be consistent with mild but not more severe disease. The means by which *F. tularensis*, which was clearly not endemic in the area, was introduced to Vermont could not be determined. Thus this investigation demonstrates both the importance of identified contact with host animals and the broad range of clinical outcomes possible following infection.

Tularemia was recognized in the Soviet Union in the late 1920s and became a significant public health problem during the years of World War II. Approximately 100,000 cases occurred between 1940 and 1945, as sanitation and population movements changed; the disease became endemic in voles and field mice. Live attenuated tularemia vaccines were developed by the Gamaleia Institute in the former Soviet Union in the 1940s, and several mass vaccination campaigns were undertaken between 1946 and 1955 (Sandström, 1994). This appears to have resulted in dramatic declines in numbers of annual cases, which are now on the order of a few hundred cases a year.

In the United Kingdom, the few reported cases of tularemia were patients who returned from travel elsewhere (Blomley, 1972; Wood, 1976), which suggests that *F. tularensis* is not enzootic in animals of the British Isles.

## Disease

### Virulence Factors of the Bacterium

The possible mechanism(s) of *Francisella* pathogenesis in animals or humans have been studied only for *F. tularensis* and the related attenuated vaccine strain (*F. tularensis* LVS). No secreted

toxins or other protein virulence factors of *F. tularensis* have been identified to date. Although all strains of *F. tularensis* are Gram negative and have an LPS, the molecule essentially lacks traditional endotoxic activity, as well as a number of immunomodulatory activities, including stimulation of tumor necrosis factor (TNF) alpha production (Sandström, 1992; Ancuta, 1996). The only consistent virulence factor identified to date is the bacterial capsule (Hood, 1977). Methods were developed for removing capsule from the virulent *F. tularensis* strain Schu S4 and were used to demonstrate that the resulting viable bacteria were less virulent (LD<sub>50</sub> reduced about 100-fold) in guinea pigs but not in mice. Electron microscopy demonstrated both the initial presence of a capsule and its removal following treatment, but it remains possible that other bacterial factors were affected by treatment. The capsule itself was neither toxic nor immunogenic, suggesting that its role in virulence was related more likely to protection of the intact bacteria against host defenses. Similarly, loss of capsule by *F. tularensis* LVS, through manipulation of culture conditions, resulted in bacteria with lowered virulence in mice (Cherwonogrodzky, 1994). More directly, a capsule-deficient mutant of *F. tularensis* LVS, derived by chemical mutagenesis, was much less virulent than the parent strain in outbred mice (Sandström, 1988). The loss of virulence was probably due to increased sensitivity of the mutant (designated LVSR) to killing by serum through activation of the classical complement pathway. Paradoxically, LVSR also was less susceptible to intracellular oxygen-dependent killing by human polymorphonuclear leukocytes (PMNs), suggesting that host recognition of the capsule also was responsible for activation of PMNs. Other research (Cowley, 1996) demonstrated that LVSR also lacked much of its LPS O antigen, which may be the basis for serum sensitivity. *F. novicida*, which apparently does not have a capsule, is nonetheless resistant to the bactericidal action of serum; in contrast to LPS from *F. tularensis*, however, LPS purified from *F. novicida* appears to be endotoxic and may retain the ability to stimulate nitric oxide production from macrophages (Cowley, 1996). The relationship, if any, of endotoxin activity to disease produced by *F. novicida* is unknown.

Disease progression and pathology have been described in monkey models of aerosol infection with both Type A (White, 1964) and Type B (Schrickler, 1972) *F. tularensis*. Even though monkeys appear somewhat more susceptible to *Francisella* infection, the course of disease in monkeys parallels that in humans. Infected monkeys exhibited an acute febrile illness with pneumonia when bacteria were introduced by aerosol. Bacteria multiplied readily in the lungs,

disseminated to regional lymph nodes, and then spread to spleen and liver; surviving animals cleared bacteria within 2 months. The cause of death in lethal cases, however, was not specifically determined, but was generally assumed to follow overwhelming bacterial burden and liver failure. Specific bacterial virulence factors responsible for disease progression, however, were not identified.

### Host Response, Immunity, and Vaccination

Since *Francisella* is a facultative intracellular bacterium, its ability to cause disease is probably most directly related to host responses following infection of macrophages and macrophage-like cells in organs of the reticuloendothelial system (spleen, lymph nodes, liver, and lung). Thus, pathogenicity is best considered in relationship to the interaction of the bacterium with host cells and tissues. The immunology of *Francisella* infection has been reviewed in some detail elsewhere (Tärnvik, 1989), and thus this section will focus on more recent developments. In general, it is worth noting that most nonvaccinated laboratory workers who handle virulent tularemia eventually become infected and suffer the disease. Dr. Francis himself had, and documented carefully, four distinct episodes of *F. tularensis* infection in the course of 20 years of studying the bacterium. This, as well as a handful of other documented cases of reinfection, raises the question of whether primary infection effectively generates specific protective secondary immunity. It is highly likely, however, that examples of reinfection are the exception rather than the rule. Other studies do indicate development of long lasting protective immunity following natural infection, and documented cases of reinfection are rare (Burke, 1977). Thus reinfection after primary infection most likely occurs only after exposure to particularly large and overwhelming doses, such as Dr. Francis might have received. For obvious practical reasons, however, researchers have preferred to use attenuated strains of *Francisella* rather than virulent strains, and thus a great deal of the current literature on *Francisella* concerns study of LVS, the live vaccine strain of *F. tularensis*. Similarly, much of what is inferred about the immunology of *Francisella* derives from study of the animal immune response to LVS.

Infection of mammals with *Francisella* initiates a cascade of nonspecific and specific host responses. As with most bacterial infections, PMNs are recruited to the site of *Francisella* infection within hours after entry. However, attenuated strains such as LVS were much more susceptible to killing by PMNs than wild-type strains, even though both are encapsulated

(Löfgren, 1983). In mice infected intravenously with *F. tularensis* LVS, bacteria initially infected Kupffer cells in the liver but also infected adjacent hepatocytes. It appeared that PMNs also recognized and killed infected hepatocytes, thus releasing bacteria for conventional phagocytosis and direct killing by PMNs (Conlan, 1992). If PMNs were not available, unrestricted bacterial growth continued in hepatocytes (which lack intracellular killing mechanisms), resulting in rapid death of the animal. Specific mechanisms of susceptibility or resistance to PMN-mediated killing of *Francisella* have not been determined, however.

*Francisella* cells that escape early killing by PMNs infect and grow readily in resident mammalian macrophages. Both *F. tularensis* LVS and *F. novicida* multiply in macrophages isolated from mice and guinea pigs. Unlike LVS, however, *F. novicida* does not grow in rat macrophages (Anthony, 1991). This apparently is because LPS stimulates rat macrophages to produce nitric oxide, which in turn ablates intracellular growth of the bacterium (Cowley, 1996). Both LVS and *F. novicida* occupy a membrane-bound compartment that appears to be a phagosome that is not fused with lysosomes (Anthony, 1991). Growth of LVS in mouse macrophages further requires an acidified compartment that permits availability of iron (Fortier, 1995). Intracellular growth further requires a two-cistron bacterial operon, designated *mgLAB*; inactivation of the operon results in decreased expression of many bacterial proteins, as well as in lost capacity to grow in macrophages and reduced virulence in mice (Baron, 1998). Surprisingly, a relatively small number of changes were found in host protein expression by macrophages following *Francisella* infection (Golovliov, 1997).

Killing of *F. tularensis* LVS by activated murine macrophages has been demonstrated. Mouse macrophages deliberately activated by treatment with interferon (IFN) gamma readily controlled the intracellular growth of *F. tularensis* LVS by both nitric oxide-dependent and nitric oxide-independent means (Anthony, 1992; Fortier, 1992; Polsinelli, 1994), although it is not completely clear whether the activity is actually bactericidal or simply bacteriostatic. Manipulations that limited availability of IFN or nitric oxide production in animals clearly increased the relative virulence of LVS, while those that activated macrophages increased host resistance (Leiby, 1992; Green, 1993). Thus, the state of host phagocyte activation has a clear effect on the severity of disease produced in mice by LVS.

Macrophage activation for control of growth probably occurs as a consequence of both innate and specific immune responses. Over the last decade, studies of several intracellular pathogens

such as *Listeria monocytogenes*, *Salmonella typhimurium* and *Leishmania* have indicated that mammals have an initial early (days to weeks) lymphocyte-independent, innate immune response phase and a later (weeks to months) lymphocyte-dependent, adaptive immune response phase of resistance to primary infection. *Francisella* appears to be no exception. Thus, both T cell deficient, athymic *nu/nu* mice (Elkins, 1993) and total lymphocyte-deficient *scid* mice (Elkins, 1996) are able to control and survive primary infection with LVS for 3–4 weeks before succumbing. As discussed, the activity of PMNs is necessary for initial survival of LVS infection in mice (Sjöstedt, 1994; Elkins, 1996). Similar to other intracellular infections, the survival of LVS infection for more than a week clearly is dependent on the production of the Th1-like cytokines IFN  $\gamma$  and TNF  $\alpha$  (Anthony, 1989; Leiby, 1992; Elkins, 1992b; Elkins, 1993; Elkins, 1996). TNF  $\alpha$ , IFN  $\gamma$ , IL-10, and IL-12 messenger RNAs and the corresponding cytokine proteins (but not IL-2, IL-3, or IL-4) were detectable in livers from mice within 24–48 hours of subcutaneous sublethal infection with LVS (Golovliov, 1995), as well as following lethal infection with virulent tularemia (Golovliov, 1996). Activated macrophages and natural killer cells, not lymphocytes, probably produce these cytokines, although direct demonstration of the cellular sources awaits further study. Further, the stimulus for activation of macrophages and natural killer cells obviously does not proceed through specific-receptor mediated recognition of *Francisella*-related molecules but presumably requires recognition of determinants common to pathogens. Bacterial cell wall polysaccharides, capsular polysaccharides, lipopolysaccharides, and unmethylated DNA containing CpG dinucleotides have been proposed as ligands for common “pattern recognition” receptors on cells such as macrophages and natural killer cells of the innate immune system (Janeway, 1992). Elucidation of such putative structures on *Francisella* awaits further study, although recent investigations indicate that bacterial DNA preparations, either oligonucleotides or genomic DNA purified from LVS, are capable of stimulating protection against lethal LVS infection in mice (Elkins, 1999b).

Another potential bridging mechanism between innate and specific immunity in *Francisella* infection has been proposed (Elkins, 1992a; Elkins, 1993; Culkin, 1997). Normal mice given a sublethal infection with LVS are, very surprisingly, strongly resistant to a second lethal LVS infection that is introduced only 2–3 days later. This resistance mechanism appears to be dependent on lymphocytes and especially B cells. Athymic *nu/nu* mice, T cell-depleted mice, and

all T cell knockout mice are able to survive double infection, but *scid* mice and B cell knockout mice are not (Elkins, 1992a; Elkins, 1993; Conlan, 1994; Culkin, 1997). This early protective response probably is not, however, related to B cell-mediated production of specific antibodies. This mechanism is nonspecific and operative in *Listeria monocytogenes* infection as well (Elkins, 1998). Because oligonucleotide DNA containing unmethylated CpG motifs stimulates protection against both lethal LVS and *Listeria* infection in mice (Elkins, 1999b), early protection may be a consequence of host recognition of released bacterial DNA shortly after infection.

Effector mechanisms of the innate immune system may be necessary prerequisites to the development of specific secondary immunity; thus macrophage activation, neutrophil activation, natural killer cell function, and cytokine production may be required not only to control initial infection but also to promote development of specific, lymphocyte-mediated immunity. It should further be remembered that in any second exposure to the bacteria, a concomitant innate immune response is again elicited, just as in primary infection, and these effector mechanisms may be quite important in both initiating and augmenting specific T cell-mediated responses. For example, it has been shown that PMNs contribute significantly to survival of secondary LVS infections in mice (Sjöstedt, 1994).

Long-term resolution of primary *Francisella* infection clearly requires the activity of conventional T lymphocytes because neither *nu/nu* mice (which have B cells) nor  $\alpha/\beta$ T cell knockout mice survive more than a month after LVS infection (Elkins, 1993; Conlan, 1994; Yee, 1996). In total T cell-deficient mice, bacterial numbers in organs quickly reach a plateau level several days after infection; this steady-state level is maintained over several weeks in apparently asymptomatic mice, until bacterial numbers begin a precipitous rise shortly before death of the animal (Elkins, 1993). The B cell knockout mice appear only slightly impaired in their ability to survive and clear a primary intradermal LVS infection (Elkins, 1999a). On the other hand, mice deficient in mature B cells and antibodies (B cell knockout mice) were 100-fold less well protected against secondary lethal challenge than were their normal counterparts. This defect in optimal specific protective immunity was readily reconstituted by transfer of primed, and to a lesser degree, unprimed B cells but not by transfer of specific antibodies (Elkins, 1999a). Thus, B cells appear to have a significant role in optimal secondary immunity to LVS in mice, through a function other than antibody production. Either CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells are apparently sufficient to effect survival, as knockout mice defi-

cient in only one T cell subset are readily able to clear primary intradermal LVS infection (Yee, 1996; see also Conlan, 1994). The specific effector function contributed by T lymphocytes, over and above cytokine production that probably contributes to macrophage activation for control of intracellular bacterial growth, remains to be revealed. The T helper cell function that is part of specific antibody production is unlikely to be critical, since CD4<sup>-</sup> knockout mice that fail to make specific IgG anti-LVS antibodies easily resolve primary (and secondary) LVS infection (Yee, 1996). In other systems, T cells have been shown to function as cytotoxic cells against bacteria-infected macrophages (Kaufmann, 1988). Inasmuch as macrophages activated by IFN  $\gamma$  appear to control (but not necessarily completely clear) bacterial numbers (Anthony, 1992; Fortier, 1992; Polsinelli, 1994), the concept of final elimination of bacteria by T cell-mediated killing is attractive. In fact, a recent study described restimulated CD4<sup>+</sup> cytotoxic T cells from peripheral blood lymphocytes (PBLs) of humans vaccinated with LVS that were specific for *F. tularensis*-pulsed monocytes (Surcel, 1991b).

The contribution of  $\gamma\delta^+$  T cells to primary infection may be minimal because the ability to survive and clear primary infection in both knockout mice deficient for the delta chain of the T cell receptor (and thus lacking  $\gamma\delta^+$  T cells) and normal mice appeared to be comparable (Yee, 1996). However, some deficiency in their ability to survive maximal secondary infection was observed: *Francisella*-immune Tcr $\delta$  knockout mice had difficulty surviving maximal lethal challenge doses, and made a reduced anti-LVS antibody response (Yee, 1996). The relationship, if any, between these two observations remains to be explored further. Dramatic polyclonal expansion of V(9/V $\delta$ 2 T cells in the peripheral blood cells of tularemia patients within 7–10 days after apparent infection has recently been observed (Sumida, 1992; Poquet, 1998). This was interpreted as suggesting that *Francisella* may possess a superantigen that stimulates a particular  $\gamma\delta^+$  T cell receptor without regard to antigen specificity (Sumida, 1992), and evidence for a non-peptide phosphoantigen derived from virulent *Francisella* (but not LVS) has been presented (Poquet, 1999). This is of particular note since other studies using a comprehensive set of *Francisella* proteins to stimulate human PBLs have not detected such a molecule (Surcel, 1990).

Humans infected with wild-type *Francisella* exhibit a T cell-dependent delayed-type hypersensitivity response within about a week of infection (Buchanan, 1971), a response that has been exploited to develop a skin test for diagnosis. Early responses of cells of the innate human

immune system, and corresponding cytokine production, to primary infection with wild-type *Francisella* or to vaccination with LVS have not been comprehensively studied. Primary T cell responses in human volunteers vaccinated with LVS have been described. By days 10–14 after LVS vaccination, in vitro proliferative responses by PBLs to killed bacteria were detected, and by day 14 IL-2, IFN $\gamma$ , and TNF $\alpha$  production (but not IL-4 production) was observed (Karttunen, 1991). Similarly, proliferative responses to killed bacteria by PBLs obtained from tularemia patients were demonstrated 2 weeks after appearance of symptoms; production of TNF $\alpha$ ; could be detected within 1 week, and IL-2 and IFN $\gamma$  (but not IL-4) were found from 2 weeks onward (Surcel, 1991a). The T cell or monocyte contribution to either proliferation or cytokine production was not specifically determined, however.

The nature of the LVS antigens recognized by mouse T cells has not been completely studied. At least one lipoprotein, designated TUL4, stimulated proliferation of T cells from vaccinated people (Sjöstedt, 1989); further, TUL4 stimulated both proliferation and cytokine secretion in T cells from immune mice (Sjöstedt, 1992b), but this protein does not appear to be immunodominant. Similarly, the human T cell response to *Francisella* appears to be heterogeneous as well as long lived. Peripheral blood lymphocytes or purified T cells (in the presence of monocytes) from LVS-vaccinated people proliferated in vitro when incubated with heat-killed bacteria (Tärnvik, 1975; Tärnvik, 1978); this proliferative response was due primarily to recognition of a wide variety of proteins, at least some of which are outer membrane proteins (Sandström, 1987; Surcel, 1989; Surcel, 1990; Sjöstedt, 1992a). Subjects who recovered from natural tularemia infection between 2 weeks and 25 years earlier also had PBLs that proliferated in response to heat-killed bacteria (Koskela, 1980; Ericsson, 1994) and to a variety of bacterial proteins as well (Sjöstedt, 1990). The T cell responses to at least four of these proteins were observed in cells from both naturally infected subjects and LVS-vaccinated subjects, suggesting that antigenic proteins important in human responses to wild-type infection also are expressed by LVS; as with mice, however, no obvious immunodominant human antigen has been found. Upon antigen stimulation in vitro, T cells from either vaccinated or naturally infected individuals produced IL-2 and IFN and exhibited upregulated IL-2 receptor expression (Karttunen, 1987). The T cell clones established from PBLs of both naturally infected subjects and LVS-vaccinated subjects have been reported and were class II restricted, CD4<sup>+</sup>, and produced IL-2 and IFN, and provided



help for in vitro antibody production (Surcel, 1989; Sjöstedt, 1990; Surcel, 1990; Sjöstedt, 1992a). Although both *Francisella*-specific proliferative and Th1 cytokine production responses by the CD45RO<sup>+</sup> (memory) / CD4<sup>+</sup> T cell subset were readily demonstrated, responses from the CD8<sup>+</sup> subset were detected as well (Sjöstedt, 1992a). T cells from LVS-immune mice also proliferated in response to heat-killed bacteria. As might be expected, the proliferative response was dependent on macrophages, and the interaction between macrophages and T cells was H-2 (and largely class II) restricted (Anthony, 1988). Although such observations suggest that CD4<sup>+</sup> T cells might be especially important in immunity to *Francisella*, the observed ability of mice totally lacking CD4<sup>+</sup> T cells to readily resolve both primary and secondary LVS infection must be remembered (Yee, 1996).

It has proven very difficult to stimulate strong protection against intracellular pathogens by immunization with killed bacteria, including in *Francisella* infection (Foshay, 1950; Clafin, 1972; Hambleton, 1974). Since the generation of a specific antibody response is very similar to both killed bacteria and live infection, it has been inferred that antibody is not sufficient for successful protection. Further, in experimental situations where little or no specific antibody is present, excellent protection can be demonstrated. Nonetheless, the antibody response to *Francisella* has been studied in some detail in both animals and humans because it has proven useful in diagnosis. In mice, IgM anti-LVS antibody responses were detected 5 days after infection, reached a peak by 2 weeks after infection, and slowly declined thereafter but persisted for more than 4 months (Rhinehart-Jones, 1994). The IgG anti-LVS antibody responses were detected 10 days after infection and peaked by about 1 month, and were maintained at a plateau level for at least 4 months. The overwhelming majority of the specific IgG response was of the IgG<sub>2a</sub> subclass, as might be expected for an infection that results in production of high levels of IFN  $\gamma$ . Much of the anti-LVS response in both mice and humans appears to be directed against the LPS of the bacterium (Viljanen, 1983). Some limited protection could be transferred to naive mice with IgG anti-LVS antibodies, but the strength of this protection was quite small compared to that afforded by immunization with live bacteria, and no protection could be transferred with IgM anti-LVS antibodies (Rhinehart-Jones, 1994). Other studies indicated that anti-LVS antibodies affected the relative distribution of bacteria in organs but not overall mortality (Anthony, 1987). In contrast, CD4<sup>-</sup> knockout mice infected with LVS produced only small amounts of IgM anti-LVS antibodies but were

able to survive a maximal secondary lethal challenge (Yee, 1996). Older literature demonstrated that serum from animals infected with wild-type *F. tularensis* was unable to transfer protection against wild-type challenge as well, and at best slightly extended the mean time to death (Allen, 1962; Thorpe, 1965). In addition, anti-*Francisella* antibodies appear to have little functional activity in that neither bacteriocidal action nor opsonization for uptake and subsequent killing by macrophages or PMNs has been readily demonstrated in vitro (Löfgren, 1983; Sandström, 1988; Rhinehart-Jones, 1994). Taken together, these results indicate that anti-LVS antibodies are neither necessary nor sufficient for optimal secondary immunity to *Francisella*.

In humans naturally infected with *F. tularensis*, specific IgM, IgG, and IgA serum antibodies appear almost simultaneously about 6–10 days after the onset of symptoms, or about 2 weeks after infection. All isotypes peak about 1 to 2 months after infection and can still be detected up to 11 years later (Koskela, 1985). However, most subjects who recovered from natural infection did not have significant amounts of specific anti-*Francisella* antibodies in their serum 25 years later (Ericsson, 1994). Similarly, in humans vaccinated with LVS, specific IgM, IgA, and IgG antibodies were detected in serum about 2 weeks after vaccination and persisted for at least 1.5 years (Koskela, 1982). There is no apparent correlation between levels of serum antibodies in human sera and protection against subsequent tularemia infection. For example, two vaccinated laboratory workers known to have high titers of agglutinating antibodies at the time of infection nonetheless suffered severe tularemia following a laboratory exposure (Overholt, 1961).

The extent of disease and its effect on military operations spurred interest in development of a tularemia vaccine in the former Soviet Union in the 1940s; in the West, efforts at vaccination began with development of killed vaccines (Foshay, 1950), but it soon became clear that killed vaccine or antigen preparations were relatively ineffective in disease prevention. Scientists at the Gamaleia Institute in the former Soviet Union apparently used several different methods to develop attenuated strains, starting with clinical isolates that were not well documented but were probably *F. tularensis* type B. The preferred method was repeated passage on artificial media, and in the mid-1940s, an attenuated strain (designated "strain 15") with greatly reduced virulence for guinea pigs and substantially reduced virulence for mice, was derived (Tigertt, 1962). After initial promising results with human use, mass vaccination of several million people were undertaken between 1946 and 1955 (Sandström, 1994). In the course of manufacturing various

lots of vaccine, however, an inverse correlation between virulence in animals and immunogenicity in people was noted. New lots that failed to kill some mice but still killed all guinea pigs (failure to kill guinea pigs was considered indicative of successful attenuation) also were thought to be less effective as vaccines. By about 1955 the original strain 15 apparently had become so attenuated that it no longer retained the necessary "residual virulence," and thus efforts to derive new strains were undertaken. Strain 15, itself, was "restored" by passage through animals to approximate its original virulence profile, and a new strain (called 155), with a phenotype in animals similar to the original strain 15, was derived through passage on artificial media. By 1960, after several decades of mass vaccination with various attenuated strains, it was stated that only a few hundred cases of tularemia occurred annually (Tigertt, 1962). More recent data are not readily available.

In 1956, a "medical mission" of United States Army doctors to the Soviet Union brought back an ampoule of tularemia vaccine from the Gamaleia Institute to the military research institute at Ft. Detrick, MD; the label on this vial suggests it was a mixture of strain 155 and the "restored" strain 15. At Ft. Detrick, culture of isolated colonies revealed that, as had previously been described in the Soviet literature, two colony morphologies could be discerned on agar plates under oblique light. Colonies of the so-called "blue variant" were small, opaque, and raised, while those of the "gray variant" were larger, transparent, and flat. Efforts to separate the two variants were not entirely successful, but organisms derived from liquid cultures of mostly blue colonies apparently were more virulent and more immunogenic for both guinea pigs and mice than were organisms derived from cultures of mostly gray colonies (Eigelsbach, 1961). For reasons that are not obvious, organisms derived from isolated blue colonies were then serially passed through mice five times; each time, virulence apparently increased, and at the last passage the bacteria were isolated from the blood of moribund animals. The final isolate was designated LVS, for Live Vaccine Strain (Eigelsbach, 1961).

The LVS is thus significantly more virulent for mice than strains used in Soviet mass vaccination programs, and, in fact, appears to have essentially full virulence for modern inbred strains of laboratory mice: a single bacterium inoculated intraperitoneally or intravenously is generally lethal for BALB/c mice. In mice as well as in guinea pigs, LVS causes a fulminant disease that is histopathologically quite similar to human tularemia. Thus, study of LVS infection of mice has been widely used as a model for study of virulent human tularemia, and in fact, as a gen-

eralized model intracellular infection (Tärnvik, 1992; Anthony, 1987; Fortier, 1991; Yee, 1996). Regrettably, because genetic systems for *F. tularensis* are in the early stages of development, the genetic basis of attenuation of LVS remains uncharacterized; thus, it is not yet possible to evaluate the relationship between LVS and fully virulent *F. tularensis* type B. However, sequencing of the genome of the virulent *F. tularensis* strain Schu 4 is near completion, and thus genomic information may soon be brought to bear on the question of the genetic basis of attenuation of LVS.

The acceptability and success in humans of LVS as a vaccine, which is not licensed for use in the United States, are uncertain. The LVS has had limited distribution in the United States as an Investigational New Drug (IND) product to qualified collaborators by the US Army Medical Research Institute in Infectious Diseases (USAMRIID) at Ft. Detrick, for use in research, vaccination of laboratory workers, and other "at risk" populations. A number of somewhat limited studies have been offered in support of its efficacy against either Type A or Type B tularemia. Remarkably, aerosol challenge studies in human volunteers were performed in the late 1950s, in which volunteers were given LVS by scarification and subsequently challenged by inhalation with virulent Schu S4 strain, the clinical Type A isolate (McCrum, 1961). Results in this limited population suggested that some protection against development of symptoms was afforded, at least at lower challenge doses; similar observations in a monkey model also have been published (Eigelsbach, 1962). An analysis of laboratory-acquired cases at USAMRIID among civilian workers before and after LVS was routinely used for vaccination was interpreted to indicate that LVS prevented typhoidal tularemia, including pneumonic tularemia, fairly well and at least ameliorated symptoms of ulceroglandular tularemia (Burke, 1977). However, the time periods studied coincided with development and introduction of considerable improvements in containment of biologicals and safe handling procedures, weakening the conclusions of this analysis. Disease in the United States is now so rare and sporadic that designing appropriate field trials of vaccine candidates is not feasible. Further, safety studies to date indicate that LVS has significant reactogenicity (as might be expected for a strain that was derived, in part, by deliberately increasing its virulence for animals), and its potential to revert to virulence for humans cannot be adequately assessed. Newer lots of LVS have varied in their relative proportions of blue and gray colonies (from 1 to 20% gray), with unknown effects on overall efficacy (Waag, 1995). Appreciation of the appearance of

blue and gray colonies as a consequence of the capacity of *F. tularensis* LVS to undergo phase variation of LPS expression (Cowley, 1996) also raises questions about difficulties in consistency of manufacture using this strain. Other efforts to develop alternate vaccine candidates may be spurred by the availability of sequence information and recent improvements in genetic systems for manipulating the *F. tularensis* genome.

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## Ecophysiology of the Genus *Shewanella*

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### Introduction

The genus *Shewanella* is comprised of more than 20 species inhabiting a wide range of environments including spoiled food, oil field wastes, redox interfaces in marine and freshwater, cold waters and sediments of the deep sea, and mesophilic ones all around the planet. Much of the recent interest in the shewanellae stems from their almost notorious abilities in the area of anaerobic respiration; these bacteria appear to be able to use nearly any electron acceptor more electronegative than sulfate, including oxygen. However, the genus is much more than just a group of respiratory specialists, as will be discussed here.

As with many contemporary genera, the *Shewanella* have experienced a rocky road to their present status, as evidenced by the species *S. putrefaciens*. Originally isolated as an active agent in food spoilage (Derby and Hammer, 1932), this organism was first called “*Achromobacter putrefaciens*,” then *Pseudomonas putrefaciens* (Shewan et al., 1960), *Alteromonas putrefaciens* (Lee et al., 1977), and finally *Shewanella putrefaciens* (MacDonell and Colwell, 1985). One notable strain of *S. putrefaciens* called “MR-1” was isolated from Oneida Lake, New York, as a metal reducer, and after genome sequencing was begun, the epithet was changed to *S. oneidensis* on the basis of molecular data, including 16S rRNA sequence analyses, DNA gyrase sequence analyses, lipid analyses, and DNA/DNA hybridization (Venkateswaran et al., 1999).

Several *Shewanella* species have been found in Antarctic Continental shelf sediments (Bozal et al., 2002) as well as in sea-ice microbial communities (adapted to grow at temperatures below 4°C) isolated from McMurdo, Antarctica (Brown and Bowman, 2001). Some of these (like *S. livingstonensis*; Bozal et al., 2002) are cultivated strains, while others (such as McMurdo.10) are as yet uncultivated and exemplify psychrophilic Antarctic strains identified only via sequence analysis of 16S rDNA (Fig. 1). Some of the psychrophilic strains are also tolerant of high

pressures, i.e., piezotolerant (Kato and Nogi, 2001). To date, only a few genera are known to have piezophilic members, and the regulation and growth under high pressure conditions of two notable species *S. benthica* (Kato et al., 1998) and *S. violacea* (Nogi et al., 1998; Nakasone et al., 1999) are under intensive study (Tamegai et al., 1998; Yamada et al., 2000).

Finally, the carbon source of many *Shewanella* strains is quite restricted, mainly fermentation end products such as lactate, some amino acids, formate, and hydrogen. Members of this group thus live a syntrophic lifestyle, in rich environments, and in association with fermentative communities that supply them the needed nutrients.

The placement of the various species within the genus *Shewanella* has largely been via the similarity of 16S rRNA sequences (Fig. 1), and while the organisms are indeed taxonomically linked by this criterion, they represent a wide range of physiological types, inhabiting many different niches (Table 1). This issue will arise several times, as it appears that the linkage on the basis of 16S rRNA may have the effect of removing virtually every physiological trait and ecological characteristic used as identifiers of the genus.

### Physiology and Habitats of the Shewanellae

The type strain of *Shewanella putrefaciens* is a Gram-negative  $\gamma$ -Proteobacteria that is motile by a single polar flagellum. It produces hydrogen sulfide when grown anaerobically on thiosulfate or polysulfide, is incapable of glycolysis and fermentation, grows on lactate, pyruvate, formate, and a few amino acids, and is capable of respiratory growth on oxygen as well as a variety of different electron acceptors, including nitrate, thiosulfate, elemental sulfur, iron oxide, and manganese oxide. This combination of traits is nearly diagnostic for many (but not all) of the shewanellae. However as more *Shewanella* species are characterized and grouped according to their 16S rDNA sequence similarities, it has become increasingly clear that these traits do not

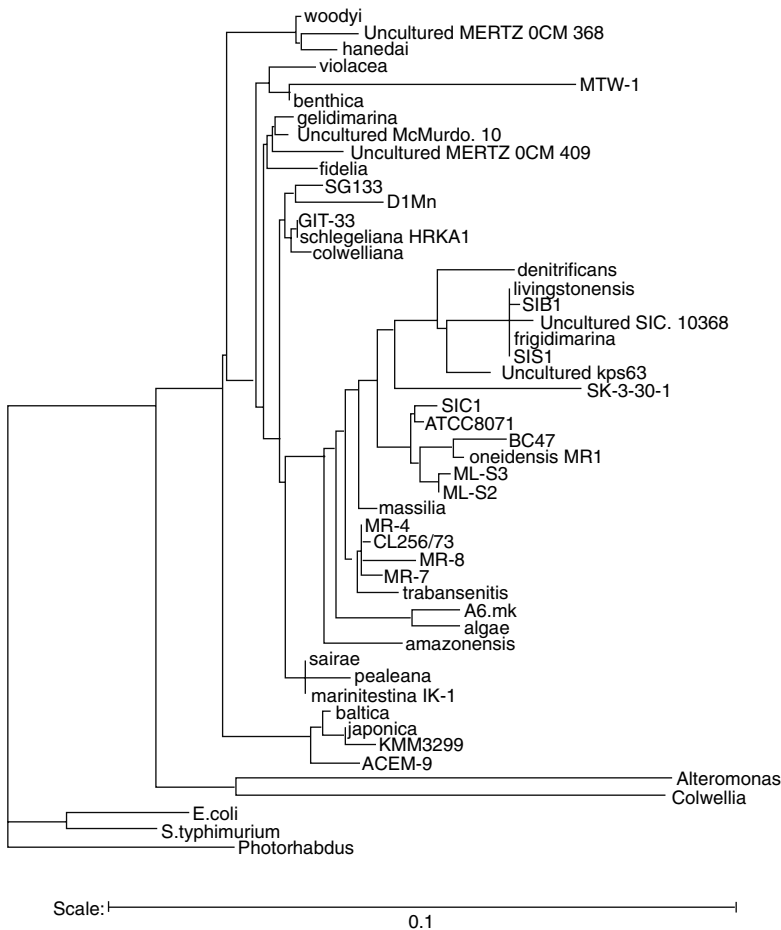


Fig. 1. Phylogenetic tree of about 40 *Shewanella* was constructed on the basis of 16S rRNA DNA sequences from both cultured clones and polymerase chain reaction (PCR) amplicons from a wide range of environments. The sequences were obtained from {the National Center for Biotechnology and Information website}. The tree was constructed using the online alignment, matrix calculation and tree building tools of the (Ribosomal Database Project (RDP-II); <http://rdp.cme.msu.edu>) at Michigan State University in East Lansing, Michigan (Maidak et al., 2001). All sequences are in the  $\gamma$ -Proteobacteria, and are linked to *Alteromonas* and *Colwellia* on one side, and *Escherichia*, *Salmonella* and *Photorhabdus* on the other. The tree was constructed from 400 nucleotide positions using the Phylip program. The maximum likelihood distance matrix was utilized with *Photorhabdus* being used as the outgroup for construction of the tree.

define all the members of the genus. Thus, to elucidate the ecophysiological features (if any) that may be common to all, the physiological traits that distinguish various *Shewanella* species are discussed before environments where they are common and abundant.

### Carbon and Energy Utilization

The carbon utilization of many isolates of *Shewanella* has been screened, and in general, ability to use complex carbon sources is absent. There is a strong preference for lactate as a carbon source, and an apparent inability to utilize many complex carbon sources under anaerobic conditions (Myers and Nealson, 1988; Venkateswaran et al., 1999). Overall, strains prefer lactate, pyruvate, or simple amino acids (Ringo et al., 1984), rarely utilize glucose, and are non-fermentative. By contrast, some of the recent psychrophilic isolates in the *frigidimarina* and other groups not only utilize glucose and other sugars, but are reported to ferment them as well (Bowman et al., 1997; Venkateswaran et al., 1999; Reid and Gordon, 1999; Kato and Nogi, 2001). Given that their metabolism is based on

the respiration of simple carbon sources (lactate, pyruvate, formate, and some amino acids) and/or hydrogen, it is reasonable that, like many sulfate reducers, the shewanellae should be syntrophic partners of fermentative microbes, driving anaerobic metabolism forward via the removal of fermentation end products (Fig. 2). In addition, under anaerobic conditions, when grown on lactate, the shewanellae release  $\text{CO}_2$  and acetate as end products. Again, the excretion of acetate is similar to that seen for many sulfate reducers, and suggestive of a lifestyle involving syntrophic partners capable of acetate utilization. Unlike the sulfate reducers, however, the shewanellae are capable of growth on a wide range of electron acceptors, including oxygen, are almost uniformly capable of the reduction of elemental sulfur, and are incapable of growth on sulfate as an electron acceptor.

Some detailed studies of carbon metabolism provide insights into the metabolism of the shewanellae, and the following statements paint a general picture of the group. Under aerobic conditions, *S. oneidensis* MR-1 utilizes a standard tricarboxylic acid (TCA) cycle for carbon metabolism, while under anaerobic conditions, other



Fig. 2. Syntrophic opportunities for the shewanellae. Under anaerobic conditions, the shewanellae utilize many of the products produced by fermentative communities, enhancing the breakdown processes. If growing on lactate, they excrete copious amounts of acetate on one hand, and reduced electron acceptors on the other. The acetate can be utilized by many anaerobic organisms, especially methanogens, while both the acetate and the reduced inorganics can be utilized by aerobic organisms.

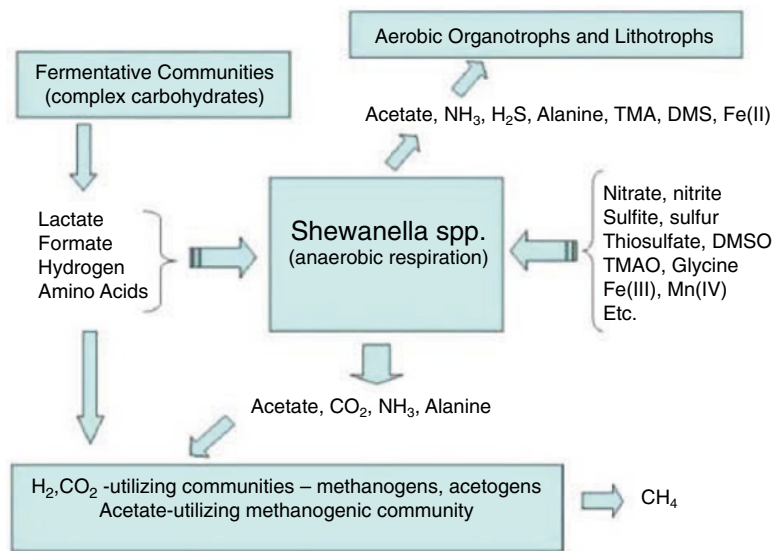


Table 1. Molar growth yields and products excreted by *S. putrefaciens* growing anaerobically with TMAO as electron acceptor.

Substrate	Growth yield <sup>a</sup>	Generation time (h)	CO <sub>2</sub> <sup>b</sup>	Acetate <sup>b</sup>	Alanine <sup>b</sup>	NH <sub>3</sub> <sup>b</sup>	%C recovered
Serine	17.5	12	2.8	0.0	0.11	0.9	104
Cysteine	17.5	12	2.7	0.0	0.10	1.1	98
Lactate	11.5	7	2.0	0.42	0.06	0.3	100
Formate	5.0	13	nd	0.0	0.0	0.2	nd

Abbreviations: TMAO, trimethylamine oxide; and n.d., no data.

<sup>a</sup>Molar growth yield as  $\mu\text{g}$  dry weight/ $\mu\text{mole}$  of substrate oxidized.

<sup>b</sup>Product excreted is expressed  $\mu\text{mol}/\mu\text{mole}$  of substrate oxidized.

Data from Ringo et al. (1984).

pathways are used (Scott and Nealson, 1994). Under anoxic conditions, during growth on lactate, acetate accumulates in the growth medium, probably via the acetyl-phosphate pathway, and is not further utilized. These results are in accord with the work of Ringo et al. (1984), who showed that with lactate, the incomplete oxidation pattern of carbon to CO<sub>2</sub> observed under anoxic conditions was inconsistent with the operation of a complete TCA cycle. It is also consistent with isotopic measurements that show a difference in carbon fractionation between aerobically and anaerobically grown cells (Teece et al., 1999).

Since growth is fastest with lactate as the carbon source, lactate is often the preferred substrate for anaerobic studies of the shewanellae. Reports of the growth on lactate vary, with some reports of anaerobic growth showing a 1 : 1 stoichiometry of lactate conversion to acetate and CO<sub>2</sub> (Lovley et al., 1989; Caccavo et al., 1992), and an earlier report that about 40% of the lactate (on a molar basis) was excreted as acetate (Ringo et al., 1984) by *S. putrefaciens* NCMB1735. The latter authors also noted that

growth on amino acids such as serine or cysteine resulted in higher molar growth yields, with no excretion of organic acids (see Table 1). Growth on formate resulted in low molar growth yields and no excretion of organic acids.

*Shewanella putrefaciens* (Lovley et al., 1989) and *S. algae* (Caccavo et al., 1992) can use H<sub>2</sub> as an energy source, and the ability of these species to remove hydrogen is such that they can out-compete sulfate reducers for hydrogen. On the basis of studies on the binding properties of uptake hydrogenases in *Shewanella putrefaciens*, at least in soil systems, Klüber and Conrad (1993) concluded that *S. putrefaciens* does not play an important role in hydrogen oxidation in that environment.

In another study of a *Shewanella* isolate (almost certainly not *S. putrefaciens*, on the basis of carbon source utilization), glucose was used as the carbon source, and nitrate as the electron acceptor (Samuelsson, 1985). While the organism showed no growth on glucose alone, it was capable of nitrate reduction, with conversion to either nitrogen or ammonia, depending on the

redox status of the medium. Such studies demonstrate the complexities of this group of organisms, and the dangers of generalization.

### Electron Acceptor Utilization

Many bacteria are somewhat versatile with regard to respiration, but few exhibit the range of activity seen in the shewanellae. The list is probably not complete, but so far, shewanellae have been shown to reduce more than 20 different electron acceptors (Table 2). This versatility is reflected in the number of cytochromes, especially *c*-type cytochromes, and in the number of cytochrome genes seen in the sequenced genome of *S. oneidensis* MR-1; [The Institute of Genomic Research [TIGR] Web site]. So far, over 40 *c*-type cytochromes are known, a great many in comparison to other bacteria of similar genome size. While the number is large,

few duplicates seem to exist, and this anomaly should be addressable via mutagenesis experiments, now underway in many different laboratories.

### Iron and Manganese Oxide Reduction

With regard to electron acceptor utilization, the shewanellae are known have a series of abilities not common to other aerobic bacteria: namely, the dissimilatory reduction of solid iron and manganese oxides, and polysulfide (see below). We say this with some caution, inasmuch as the dissimilatory reduction of metals was shown many years after the deposition of the type strain of this organism in the American Type Culture Collection, and the same could easily be true of other groups of bacteria already in “captivity.” The ability to reduce iron was reported many years ago, even for some of the strains of *S. putre-*

Table 2. Electron acceptors utilized by shewanellae isolates.

Electron acceptor	Reduction products <sup>a</sup>	References <sup>b</sup>
Oxygen	H <sub>2</sub> O	Derby and Hammer, 1931
Nitrate	NO <sub>2</sub> <sup>-</sup> , NO, N <sub>2</sub> O, N <sub>2</sub> , and NH <sub>4</sub> <sup>+</sup>	Samuelsson, 1985 Krause and Nealson, 1997
Nitrite	NO, N <sub>2</sub> O, N <sub>2</sub> , and NH <sub>4</sub> <sup>+</sup>	Samuelsson, 1985
Mn(IV) solid	Mn(II) soluble	DiChristina et al., 1988 Myers and Nealson, 1988
Mn(III) chelate	Mn(II) soluble	Kostka et al., 1995
Mn(III) solid	Mn(II) soluble	Larsen et al., 1998
Fe(III) chelate	Fe(II) soluble	Arnold et al., 1988 Myers and Nealson, 1988
Fe(OH) <sub>3</sub> ferrihydrite	Fe(II) soluble	Roden and Zachara, 1996 Urrutia et al., 1998, 1999
FeO(OH) goethite	Fe(II) soluble	Roden and Zachara, 1996 Urrutia et al., 1998, 1999
Fe <sub>2</sub> O <sub>3</sub> hematite	Fe(II) soluble	Roden and Zachara, 1996 Urrutia et al., 1998, 1999
Fe <sub>3</sub> O <sub>4</sub> magnetite	Fe(II) soluble	Kostka and Nealson, 1995 Dong et al., 2000
Fe(III) clay smectite	Fe(II) soluble	Kostka et al., 1996, 1999a, b
SO <sub>3</sub> <sup>-2</sup>	H <sub>2</sub> S	Perry et al., 1993
S <sub>2</sub> O <sub>3</sub> <sup>-2</sup>	H <sub>2</sub> S	Perry et al., 1993
S <sup>0</sup>	H <sub>2</sub> S	Perry et al., 1993 Moser and Nealson, 1996
U(VI) soluble	U(IV) solid	Wade and DiChristina, 2000 Fredrickson et al., 2000
Cr(VI) soluble	Cr(III) solid	K. H. Nealson, unpublished observation
Selenite	Se <sup>0</sup> solid	Taratus et al., 2000
Arsenate	Arsenite, As <sup>0</sup>	D. K. Newman, personal communication
Tc(VII) soluble	Tc(IV) solid	Wildung et al., 2000
Iodate	Iodide	Ferrenkopf et al., 1997
Trimethylamine- <i>N</i> -oxide	Trimethyl amine	Ringo et al., 1984
Dimethylsulfoxide	Dimethylsulfide	Myers and Nealson, 1988
Fumarate	Succinate	Myers and Nealson, 1988
Glycine	Alanine	Myers and Nealson, 1988

<sup>a</sup>The products of reduction are the primary products. For the metals, a variety of products are formed, depending on environmental conditions.

<sup>b</sup>As near as possible, these represent the first reports of specific electron acceptor utilization, and other useful references. The list is not complete.

*faciens* (Obuekwe et al., 1981; Obuekwe and Westlake, 1982; Semple and Westlake, 1987), but the unequivocal evidence that metal reduction was coupled to cellular metabolism and growth in the shewanellae was not reported until the late 1980s (DiChristina et al., 1988; Myers and Nealson, 1988). Since then, many metal reducers in a wide range of bacteria and archaea have been reported to be dissimilatory metal-reducing bacteria (DMRB). Now, reduction of a wide range of iron oxides as well as iron rich clays has been shown (Table 2).

The ability to reduce metal oxides is one that was doubted for many years because of the fact that most of the oxidized metals available in abundance in nature are solids (iron and manganese oxides form a range of insoluble forms, and virtually no soluble forms with the exception of organic ligands of Mn[III] and Fe[III]). The existence of the solid oxides within a metal's cycle provides an interesting twist for two reasons. First, in stratified water bodies like meromictic lakes or fjords, because the oxidized solid forms of iron and manganese sink, the cycling of these metals is in effect an oxidant pump, delivering oxidizing equivalents to the deeper waters. It is essentially a gravity-driven redox cycle (Fig. 3). In sedimentary environments, on the other hand, the layers of oxidized metals are fixed into the soils or sediments, and if not completely reduced by the next cycle of nutrients (usually

on a yearly cycle in temperate zones), they can leave a layer of oxidized iron and/or manganese in the sediments—a record of previous microbial metal cycling in the soils.

One of the predictable outcomes of interacting with solid surfaces as oxidants is that the reaction rates can be a function of surface area rather than cell number. Burdige et al. (1992) studied reduction of a series of manganese oxides by various shewanellae, and have shown that surface area of the oxides is of great importance with regard to predicting rates of reaction. In fact, some “aged” oxides like pyrolusite are virtually unavailable to the shewanellae as electron acceptors, owing likely to a combination of low surface area and high crystallinity (Burdige et al., 1992). Similar conclusions have been reached with studies of iron oxides by a number of different workers (Roden and Zachara, 1996b; Urrutia et al., 1998), concluding that surface area and crystallinity are the major factors that determine the rates of reduction. Such information is of more than idle interest to those wishing to study these processes in that positive results are difficult to obtain if highly crystalline oxides of low surface area are used for experiments, especially for initial enrichments.

Another important part of the metal cycle is the ability of metal oxides to act as natural ligands for other metals. This is particularly true for manganese oxides (notorious for their ability

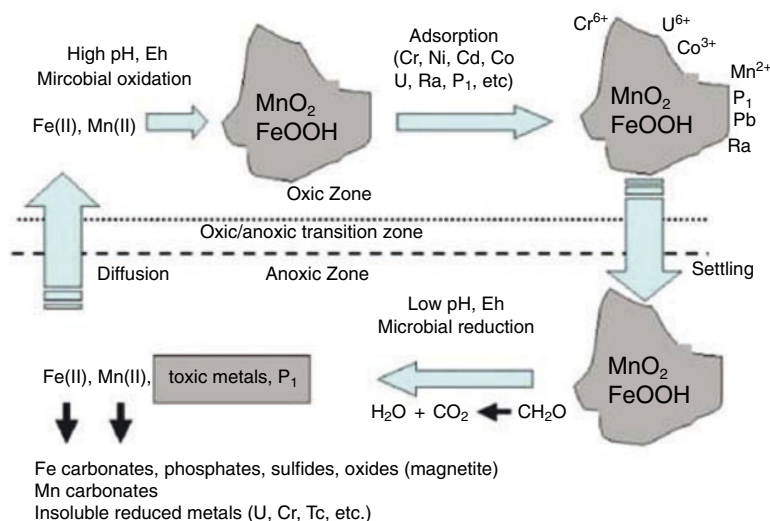


Fig. 3. The major processes occurring during the biogeochemical cycling of iron and manganese oxides across the oxic/anoxic boundary. Under conditions of high Eh and pH, iron and manganese are oxidized, and form hydrous metal oxides and oxyhydroxides. These fresh oxides are strong ligands for a variety of transition metals and actinides, as well as some heavy metals, and phosphorous, so that the insoluble oxides act as sinks for these other metals, transporting them to sedimentary environments via precipitation. In the anoxic zone, reduction occurs, either spontaneously via Eh/pH effects, or via microbial catalysis. This results in the release of the bound metals as well as Fe(II) and Mn(II), and the recycling of iron and manganese via diffusion to the oxic zone. In addition, secondary metals can be formed, depending on the chemistry of the anoxic environment in which the reduction is occurring.

to bind cations of all kinds) and for iron oxides, which strongly bind phosphate. Reduction of these oxides can then result in the release of a number of other metals into solution. Phosphate can be beneficial, and in oxic soils or marine environments, the availability of iron-reducing bacteria as symbionts may well be an important strategy in avoiding phosphorous limitation (Fig. 3). A recent proposal suggests that there have been major times in the Earth's history when productivity of the oceans was limited by phosphate, and that the relief of this limitation was achieved via iron reduction (Bjerrum and Canfield, 2002). In other cases of metal reduction, potentially toxic metals may be released, causing potential problems, or at least allowing them to diffuse to other sites. An example of such a case is shown in Fig. 4, where the cycle of uranium (U) in the Mississippi Delta is shown to be strongly regulated by the reduction of Mn oxides (Nealson et al., 2002).

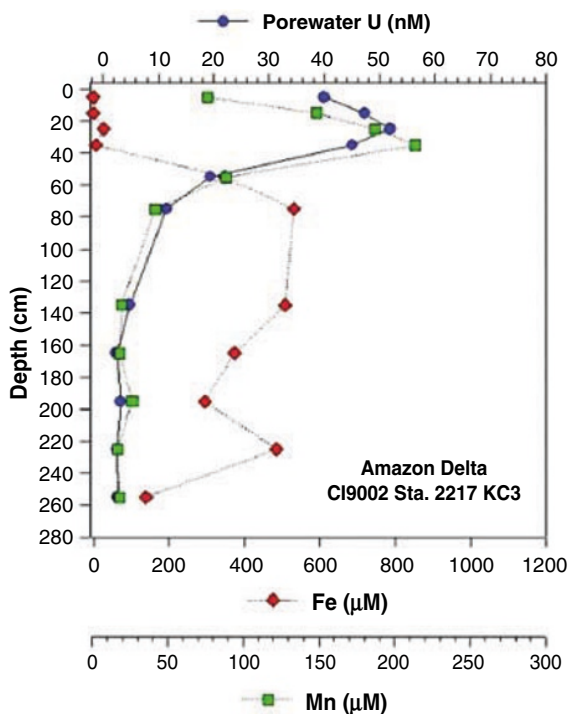


Fig. 4. Release of uranium during metal reduction. This profile plotted from data obtained during a cruise to the Amazon Delta region (Nealson et al., 2002), shows the porewater profiles of soluble iron, manganese and uranium. As seen, the uranium, which is in its soluble, oxidized (U[VI]) state, tracks almost perfectly the profile seen for Mn. This is because as the sedimentary Mn oxides are reduced by dissimilatory metal reducing bacteria (DMRB), the uranium, which is loosely bound to the Mn oxides is released into the sediments. In contrast, there appears to be no relationship between the profile of soluble iron and uranium.

The enzymatic mechanism(s) leading to metal reduction is not yet fully elucidated (Newman, 2001), and will not be reviewed here. Suffice it to say that many advances have been made, including investigations of many enzymes, genes coding for these enzymes, regulation of metal reduction, and the possibility that extracellular electron shuttles are utilized for metal reduction. From the work of several laboratories, some of the enzymes involved in metal reduction are clearly located on the outer cell wall of the shewanellae (Myers and Myers, 1992b; Myers and Myers, 1993a; Myers and Myers, 1998; Myers and Myers, 2001; Beliaev and Saffarini, 1998; Field et al., 2000; Gordon et al., 2000; Beliaev et al., 2001; DiChristina et al., 2002). Given the early reports that the reduction of solid metal oxides required cell contact with the oxides, and the more recent reports that reduction of iron requires cell adhesion (Caccavo, 1999; Das and Caccavo, 2000; Das and Caccavo, 2001), the location of such enzymes is reasonable, although the mechanism(s) whereby energy is conserved during reduction will of necessity be different from the more standard redox systems of other bacteria.

The role of quinones is not entirely elucidated, but through the work of several laboratories, it is clear that under anaerobic conditions, menaquinones are synthesized and play a major role in metal reduction. Mutants deficient in quinone production are defective in metal reduction as well as the reduction of several other substrates (Myers and Myers, 1993b; Saffarini et al., 2002). The shewanellae, along with a few other species of primarily aerobic bacteria produce methylmenaquinones (MMKs; Venkateswaran et al., 1999), and the presence of specific MMKs has been proposed as a taxonomic trait of these groups (Akagawa-Matsushita et al., 1992). Interest in quinone physiology has recently increased with the reports of Newman and Kolter (2000) and Hernandez and Newman (2001) that surface contact is not always necessary for metal reduction. Instead, they postulate that external "electron shuttles" in the form of quinones are utilized for metal reduction. These workers have shown that it is possible to isolate *S. oneidensis* mutants that cannot reduce solid iron unless supplied with quinones of the proper type. The resolution of this issue has important implications for the ecology of the metal-reducing shewanellae, as their interaction with surfaces may be a significant part of their strategy for survival and success. If surface interaction is unnecessary, or necessary only at certain times or in certain niches, these details of metal reduction will need to be determined to understand the ecophysiology of this group.

## Environmental Effects of Fe and Mn Oxide Reduction

In carbon-rich sediments, metal reduction can be a major process and account for much of the carbon cycling. As shown in Table 3, a number of environments are now characterized where from 20% to virtually all of the carbon oxidized can be accounted for by metal reduction (see Thamdrup, 2000a for a comprehensive review). In general, metal cycling can play a major role in carbon-rich aquatic sediments, and even in marine systems, where it is usually assumed that sulfate reduction dominates. This view of the role of metals in the overall carbon cycling of sedimentary environments constitutes a major change in thinking, which has taken place over the last decade. Prior to this, the solid metals were considered to be rather inactive, and their role in carbon mineralization at the most was considered to be stoichiometric. It is now clear from work like that cited in Table 3 that metals may turn over many times before being buried, and can be a dynamic component of many sedimentary environments.

In addition to the cycling of organic carbon, metal reduction has physical effects—solid metal oxides are solubilized and allowed to diffuse away so that the very texture of an environment is changed. A laboratory example (see Fig. 5) is a culture of *S. oneidensis* solubilizing the solid  $MnO_2$ . The complete solubilization of metal oxide occurs in a few days, and in systems where oxygen is available, Mn reoxidation can occur rapidly, resulting in a cyclic oxidation similar to that shown in Fig. 2. Another physical effect is that seen with smectite clays, which upon iron reduction can change their swelling capacity, charge, and other physical properties resulting in major changes to the soil environment (Kostka et al., 1996; Kostka et al., 1999b; Kostka et al., 1999c).

Finally, as noted in Fig. 3, metal reducing bacteria interact via the formation of new minerals

upon reduction of iron and/or Mn oxides. When iron oxides are reduced, the fate of the Fe(II) is specified by the environment as much or more than by the bacterium. Thus, one can alter the iron to form siderite (iron carbonate), magnetite (a mixed phase iron oxide), pyrite or other iron sulfides, or vivianite (iron phosphate), and perhaps many other products, simply by growing the cells in media with different buffer systems (Nealson and Saffarini, 1995a; Roden and Zachara, 1996b; Urrutia et al., 1998; Urrutia et al., 1999; Bjerrum and Canfield, 2002). The introduction of Fe(II) or Mn(II) and reformation into new mineral forms is of course potentially very important with regard to the ecosystem dynamics.

## Reduction of Sulfur Compounds

The shewanellae are unusual, perhaps unique, as aerobic bacteria, in their ability to grow at the expense of elemental sulfur (in the form of polysulfide; Moser and Nealson, 1996). They produce hydrogen sulfide ( $H_2S$ ) from thiosulfate, sulfite, or polysulfide, and are remarkably resistant to  $H_2S$  (Perry et al., 1993). As such, they are as “comfortable” at interfaces with sulfate-reducing bacteria as they are with aerobic heterotrophs, and span a very wide range of ecological niches in layered communities like those of the Black Sea (Nealson et al., 1991). Very little work has been done with the sulfur-reducing systems in the shewanellae, and sulfur reduction (especially in marine systems) may be one of the potentially important roles of such organisms.

## Reduction of Nitrogen Compounds

The shewanellae are capable of reduction of nitrate to nitrite, nitrous oxide, ammonium, or dinitrogen, depending on conditions (Samuelsson, 1985). Brettar and Höfle (1993) identified *S. putrefaciens* (now named “*S. baltica*”; Ziemke et al., 1998) as the major organism present and

Table 3. Oxidation of organic carbon by the reduction of Mn and/or Fe.

Location <sup>a</sup>	% of total C oxidation due to metal reduction	Metal used as electron acceptor	Reference
Panama Basin	~100	Mn	Aller, 1990
Skagerrak	~90	Mn	Canfield et al., 1993a,b
Black Sea Shelf	23–73	Mn	Thamdrup et al., 2000
Oneida Lake	25–75	Mn	Aguilar and Nealson, 1990
Talladega wetland	38–55	Fe	Roden and Wetzel, 1996
Amazon shelf	>40	Fe	Aller et al., 1991
Skagerrak	32–51	Fe	Canfield et al., 1993a,b
Gulf of Trieste	14–73	Fe	Hines et al., 1997
Norwegian fjords	10–26	Fe	Kostka et al., 1999
Chile margin	12–29	Fe	Thamdrup and Canfield, 1996

<sup>a</sup>For details of study sites, temperatures, and more examples, see Thamdrup (2000).

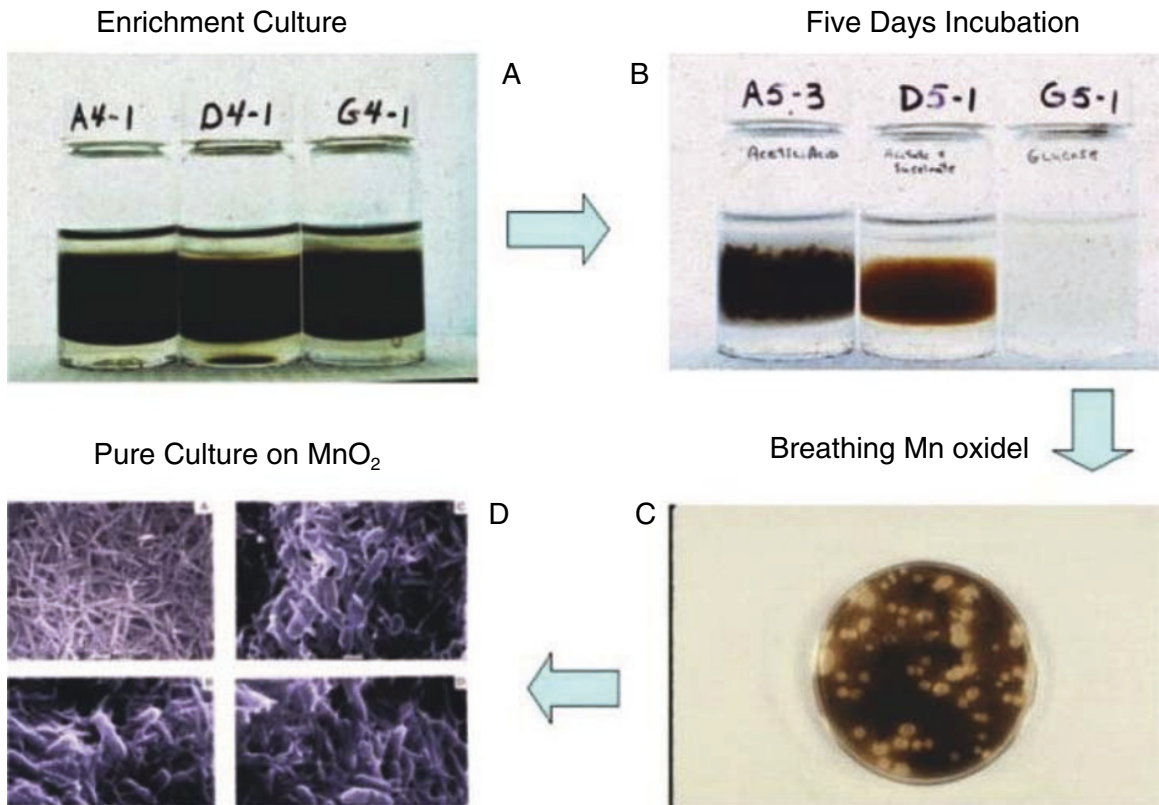


Fig. 5. *Shewanella oneidensis* and manganese reduction. A. Enrichment cultures set up for isolation of dissimilatory metal-reducing bacteria (DMRB)—soft agar vials or tubes containing  $\text{MnO}_2$  as the only electron acceptor. Enrichments are covered with a layer of mineral oil to keep them anaerobic, and contain one or more carbon sources. B. Enrichments after several days of growth. Poisoned or uninoculated samples show no change, while those with inocula show variable results depending on the carbon sources added. Here acetate, acetate plus succinate, and glucose utilized as carbon sources are seen. The glucose has resulted in metal reduction owing to acid production. C. Enrichments on lactate-media plates are incubated with an overlay of  $\text{MnO}_2$ -containing top agar. Clearings in the top agar layer are due to reduction of the  $\text{MnO}_2$  by dissimilatory metal-reducing bacteria (DMRB). D. Scanning electron microscopic views of pure DMRB (*S. oneidensis*) cultures growing on  $\text{MnOOH}$  surfaces. Upper left = uninoculated surface, Others = bacteria attached to surface.

responsible for organic carbon cycling and nitrous oxide production at the oxic/anoxic interface in the Central Baltic Sea, and proposed that it was a major factor in the carbon/nitrogen cycle in this marine environment. As with the sulfur compounds, much work is yet to be done on the physiology of nitrogen oxide reduction by the shewanellae, but the abundance of these organisms at nitrate-rich interfaces in the Black Sea (Nealson et al., 1991) and other marine environments, as well as the above-cited work leads to the conclusion that shewanellae will have major environmental impacts on the nitrogen cycle and its coupling to the oxidation and cycling of organic carbon.

### Reduction of Other Inorganic Electron Acceptors

As shown in Table 2, the shewanellae are capable of reducing a seemingly endless array of electron

acceptors down to the level of sulfate, but not including either sulfate or  $\text{CO}_2$ . This includes oxidized forms of chromium, selenium, arsenic, uranium and technetium (Fredrickson and Gorby, 1996; Fredrickson et al., 2000; Wade and DiChristina, 2000; Wildung et al., 2000). The ecological importance of these reactions is unknown, but they all have potential bioremediation uses, as reduction of these toxic metals almost always alters their solubility.

### Types of Habitats

Considering the above traits, the kinds of niches where shewanellae might be expected to be found include environments: 1) that are energy rich in which fermentation is occurring and energy is continuously being deposited via sedimentation; 2) where the redox conditions might change rapidly and shift the dominance of electron acceptors, including between oxic and

anoxic states; and 3) in which other partners are present to remove the acetate produced via anaerobic respiration. These general notions fit well with the recorded isolations of shewanellae from a wide variety of mostly energy-rich niches appropriate for multispecies anaerobic metabolism, and many of which are subject to periodic redox changes.

To some extent, the ability to use the products of other anaerobes and aerobes, and a wide variety of different electron acceptors, and the ability to sense and move towards electron acceptors, are all traits that make the shewanellae ideal gradient organisms (microbes not only tolerant of, but well suited to the “gradient lifestyle”) and would appear to be best adapted to syntrophic coexistence with other bacteria. Figure 6 is a schematic showing where the shewanellae have been found; some of these niches will be discussed in detail below. Of particular note is the expected (and observed) ability of the shewanellae to utilize oxygen, and thus not only tolerate rapid changes in redox status of the environment, but also thrive in them.

**REDOX-RELATED HABITATS** Considering the above, it is reasonable to expect that the redox interfaces, which exist nearly everywhere that biomass is produced and cycled, in the habitats occupied by the shewanellae are truly cosmopolitan, and include a wide variety of freshwater and marine niches, especially energy-rich and dynamic environments. Particularly in marine stratified communities, these versatile bacteria comprise major parts of the populations at

redox interfaces (Brettar and Höfle, 1993; Ziemke et al., 1997; Ziemke et al., 1998). For example, in the Black Sea, in which redox strata occur at intervals of many meters, viable counts of bacteria revealed shewanellae accumulated at anaerobic interfaces, and were below limits of detection in the intervening depths (Nealson et al., 1991). Similarly, Höfle and Brettar (1996) have reported the abundance of shewanellae at redox interfaces in the stratified water column of the North Sea, consequently labeling them with the very appropriate epithet “interface organisms.”

As environments become more oligotrophic, the occurrence and abundance of shewanellae tends to decline. For example, shewanellae are typically absent in low energy groundwater or vadose zone environments with constant conditions and low nutrient flow. Studies of North American lakes have supported this view (DiChristina and DeLong, 1993; MacGregor et al., 1997; Stein et al., 2001). In Green Bay or Oneida Lake sediments where sediment deposition rates are high, shewanellae are abundant. However, as one moves to environments with successively lower fluxes of organic carbon, e.g., to deeper parts of Green Bay and then to Lake Michigan, the shewanellae disappear and are replaced by other metal-reducing microbes (predominantly bacilli; C. Aguilar and K.H. Nealson, unpublished observation).

**PATHOGENIC HABITATS** Several different shewanellae have been implicated as opportunist pathogens of humans (Khashe and Janda, 1988; Kim et al., 1989; Nozue et al., 1992; Dominguez et al., 1996; Holt et al., 1997; Iwata et al., 1999), and extensive work by Gram and colleagues (Gram et al., 1999; Vogel et al., 2000) has shown that the clinical isolates are virtually identical to those seen in environmental samples. The predominant organism capable of human infection is *S. algae*, which is also a remarkably versatile redox-active organism isolated from a variety of marine environments. This species, implicated in a case of human septicemia in Japan (Iwata et al., 1999), may well possess characteristics making it particularly suited as a human secondary or opportunistic pathogen. To this end, the shewanellae have been shown to produce only hydroxamate-type siderophores (Gram, 1994), one of which has been structurally characterized and designated as “putrebactin” (Ledyard and Butler, 1997). The siderophores of *S. algae* have not been characterized, but a slight difference in structure or regulation from that of *S. putrefaciens* could easily account for the apparently more virulent nature of this species. Only one isolate has been implicated in symbiosis with eukaryotic organisms: *S. pealeana* (Leonardo et

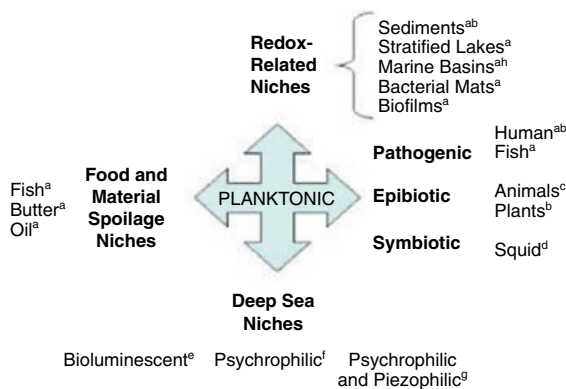


Fig. 6. The major niches of the shewanellae. As with many aquatic microbes, the niche often sampled is planktonic, but the niches are diverse. Superscripts have been added to indicate the major groups of the shewanellae found in each niche subdivision. These are a = *S. putrefaciens*, *S. oneidensis*; b = *S. algae*; c = *S. sairae*, *S. schlegeliana*, *S. marinintestina* ??; d = *S. pealeana*; e = *S. woodyi*, *S. hanedai*; f = *S. frigidimarina*, *S. gelidimarina*; g = *S. benthica*, *S. violaceae*; h = *S. baltica*; i = ??; j = *S. schlegi*, etc.

al., 1997), which was isolated from the nidamental gland of a marine squid. Several others, however, have been isolated as epibionts from both plant and animal materials (see below).

**FOOD SPOILAGE HABITATS** *Shewanella* species as a food spoilage organism was first reported by Derby and Hammer (1931), and the number of such reports has continued to grow since then. The shewanellae become dominant organisms on stored marine fish materials, and are probably one of the major organisms responsible for odor production via both trimethylamine oxide (TMAO) reduction to trimethylamine, and production of hydrogen sulfide (Levin, 1968; Gram et al., 1987, Gram et al., 1990; Jorgensen and Huss, 1989; Stenstrom and Molin, 1990; Subasinghe and Shariff, 1992; Gram, 1993). It seems likely that the rich nature of the food environment provides the type of habitat needed for the shewanellae, and the abundant TMAO (and sulfur compounds) in marine fish may make these habitats ideal for the shewanellae. Of particular interest are the species that can grow well near 0°C, such as *S. frigidimarina* and *S. gelidimarina* (Fig. 1), and their potential role in food quality degradation. Many of these species are capable of a much wider range of carbon metabolism than that normally seen in the mesophilic shewanellae (Bowman et al., 1997; Bowman et al., 2000).

**DEEP SEA, PSYCHROPHILIC, EPIBIOTIC AND BIOLUMINESCENT SHEWANELLAE** Very little is known about the ecophysiology of the bacteria that inhabit these niches, and virtually nothing is known about the nature of the microniches or the numbers of organisms present. Recent work by Nichols et al. (2000), and Bowman et al. (Bowman et al., 1997; Bowman et al., 2000) suggests a critical feature of the deep sea cold- and pressure-tolerant microbes is their ability to produce eicosapentaenoic acid (EPA). However the strains adapted to pressure and cold-temperature appear to be biochemically and phylogenetically related. Also, these traits appear to be associated with the ability of shewanellae to survive as epibiotic strains in the tissues of various marine animals.

Several epibiotic strains of *Shewanella* have been identified and characterized including *Shewanella marinintestina*, *S. schlegeliana* and *S. sairae* from intestines of various marine animals (Satomi et al., 2003). While *S. sairae* and *S. marinintestina* cluster closely with *S. pealeana*, *S. schlegeliana* clusters closely with *S. cowelliana*. Biochemical analysis of the epibiotic strains indicates that EPA can make up a significant portion of their unsaturated lipids (Satomi et al., 2003). Though EPA may play an important role in the

colonization of the host, there is no strong support for such a conjecture at this point. In fact there is strong evidence that the presence of EPA may be related to temperature adaptation. Recently it was demonstrated that when grown at 28°C, *Shewanella pealeana* appeared not to produce detectable levels of EPA (Leonardo et al., 1997). However, when grown at a lower temperature (20°C), the same strain was shown to produce significant levels of EPA (Satomi et al., 2003). Therefore the presence of EPA may have little direct influence on the host-epibiont relationship.

The trait of bioluminescence is found in only two species of shewanellae, *S. hanedai* and *S. woodyi*, both isolated from deep cold water, and both capable of growth at 4°C. The ability to emit visible light is consistent with location in the dark, deep sea, and given the association of *S. pealeana* with squid, a symbiotic luminous habitat for these species might be imagined. Both the mechanism of the light emission (bacterial luciferase), and wavelengths emitted are similar to that seen in the luminous vibrios and photobacteria. However, these two species were isolated as planktonic bacteria and nothing else is known of their luminous niches or ecophysiology. One item of interest is that these bacteria lack the capacity for widespread redox chemistry so characteristic of most of the other shewanellae.

Kato and Nogi (2001) have proposed that the shewanellae be divided into two groups on the basis of ability to adapt to and grow in the deep sea. This would put several closely related organisms like *S. benthica*, *S. violacea*, *S. pealeana*, *S. gelidimarina*, marine epibionts, and the luminous shewanellae into a separate subgroup, with *S. violacea* and *S. benthica* being placed in a branch with the piezophilic strains. As the authors note, this division has some difficulties, e.g., those tying the 16S rDNA sequence data to the physiological traits of the shewanellae in general. Nakasone et al. (K. Nakasone et al., personal communication) are in the process of completing the genomic sequence for one of these bacteria. As discussed later, this sequence may provide major insights into the differences among the shewanellae, as both the 16S rRNA sequence (Fig. 1) and deep-sea niche of *S. benthica* and *S. oneidensis* MR-1 are widely separated.

## Isolation and Characterization of the Shewanellae

Several notable features of most shewanellae can be used for enrichment: 1) the metabolism of lactate via anaerobic respiration of several



electron acceptors including thiosulfate, nitrate and hydrous metal oxides; 2) aerobic metabolism; and 3) the production of H<sub>2</sub>S from thiosulfate during anaerobic growth. Along with a few other groups of metal-reducing bacteria, the shewanellae can also use molecular oxygen as electron acceptors. Thus the aerobic growth cycle can be used to speed up initial enrichments, although the shewanellae are so abundant in many metal-reducing environments, and grow so well in enrichment cultures, that such procedures are not necessary. However, for teaching and demonstration purposes, enrichment cultures can be achieved with any of several general media as follows: growing organisms anaerobically using lactate (20 mM) as the carbon source, and nitrate or fumarate (5–50 mM) as electron acceptor, then transferring them first to aerobic medium with lactate as carbon source, second to anaerobic medium with thiosulfate (50 mM) as electron acceptor, and lactate as electron donor, and lastly to anaerobic medium with iron or manganese oxide (solids at approximately 100 mM) as sole electron acceptor, and lactate as electron donor. At each stage of the enrichment, the cells are plated out on metal overlay plates (see below) and the colonies are picked and checked for H<sub>2</sub>S production. Thiosulfate-containing medium with Fe(II) is used so that any H<sub>2</sub>S formed through thiosulfate reduction results in the formation of a black (iron sulfide) precipitate. This trait is nearly diagnostic for the shewanellae.

### Precautions and Recommendations

- 1) Mn oxides are easy to reduce and may give false positives. Because Mn oxides are more quickly reduced, demonstration of Mn oxides reduction is a good teaching tool
- 2) Addition of reductants to the medium (e.g., cysteine) to lower the redox, or production of organic acids, H<sub>2</sub>S, or Fe(II) can result in Mn(IV) reduction
- 3) Fe oxides are more stable, and difficult to reduce abiotically
- 4) Highly crystalline metal oxides (Fe or Mn) purchased from most supply houses have low surface area and low reactivity. Thus, make your own
- 5) Metal oxides (especially Mn oxides) stored in liquid suspension tend to change (coagulate and “age”) with time, leading to differences between experiments and ultimately to very unreactive metal oxides
- 6) Fe(III) can be prepared in a number of soluble forms, using chelators such as NTA (nitriloacetic acid), citric acid, ammonium citrate, or others. Such approaches reduce the

complexities seen when solid surfaces are added to experiments

### Preparation of Manganese and Iron Oxides

**MANGANESE OXIDES** Mn oxides are in general very easy to prepare (see Kostka and Nealson, 1997). There are a number of methods, all of which produce acceptably active, amorphous or poorly crystalline MnO<sub>2</sub>. One method is as follows:

- 1) Dissolve 8 g of KMnO<sub>4</sub> in 200 ml of distilled water
- 2) Heat to 90°C while stirring continuously
- 3) Add 10 ml of 5 N NaOH
- 4) In a separate flask, dissolve 15 g of MnCl<sub>2</sub> · 4H<sub>2</sub>O in 75 ml of distilled water. Add this solution slowly to the basic permanganate solution. This results in a precipitate being rapidly formed
- 5) Continue heating and stirring for one hour
- 6) After cooling, the MnO<sub>2</sub> is washed several times by centrifugation and resuspension in distilled water
- 7) After the final resuspension, the solid is freeze dried and stored as a fine dried powder

This material is referred to as “δMnO<sub>2</sub>” or “vernadite” (Balistrieri and Murray, 1982), but unless the mineralogy is determined, it is more appropriate to refer to it as “amorphous MnO<sub>2</sub>” or “manganate,” a generic term for Mn oxides of the formula MnO<sub>2</sub>.

If weighed subsamples are stored, they can be rehydrated to similar concentrations, and the reactivity of the manganate is reproducible. Samples can also be autoclaved before lyophilization, and stored as sterile dry subsamples for later use. A slight variation of this method is presented in Kostka and Nealson (1997).

**IRON OXIDES** Iron oxides range from amorphous Fe(OH)<sub>3</sub> (ferrihydrite or rust), to highly crystalline forms such as goethite, and magnetite. Like the Mn oxides, the iron oxides can change with time, forming a wide range of intermediate complex forms.

A complete treatise on the preparation and properties of iron oxides is available (and recommended) if detailed knowledge of iron oxides is required (Schwertmann and Cornell, 1991). Because ferrihydrite is commonly used in enrichments, one of the many ways of preparing it (Schwertmann and Cornell, 1991) is given below.

- 1) Dissolve 40 g of Fe(NO<sub>3</sub>)<sub>3</sub> · 9H<sub>2</sub>O in 500 ml of distilled water
- 2) Add KOH (approximately 330 ml of a 1.0 M solution) to bring the pH to 7.8. As pH approaches 7.8, add last few ml dropwise with constant monitoring and stirring

- 3) Ferrihydrite suspension is centrifuged and washed six times in distilled water
- 4) Use on day of preparation, as goethite or hematite will form with age
- 5) Ferrihydrite can be lyophilized and stored dry with only minor alterations

This method gives a good general, high surface area, reactive iron oxide for enrichment cultures.

### Basal Medium (M1)

M1 basal medium is used, but simply using complex medium (peptone-yeast extract media of almost any type) with lactate as the carbon source will suffice for general isolation of shewanellae. It is critical that lactate be used as the carbon source for enrichment. As far as is known, all *Shewanella* species can use lactate, which reduces competing contaminants, and also diminishes any nonspecific metal reduction due to organic acid production, etc.

#### M1 Medium

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.19 g
PB	15 ml
BSS	100 ml
Distilled water	875 ml
MSS	0.1 ml
NaHCO <sub>3</sub>	10 ml
AA	10 ml
Carbon substrates	variable
Electron acceptors	variable

For one liter of medium, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> is dissolved in a solution containing phosphate buffer (PB), basal salts solution (BSS), and distilled water in a 2-liter Erlenmeyer flask. Then, 0.1 ml of trace metals supplement (MSS) is added before adjusting to pH 7.0 with 10 N NaOH or 10 N HCl as needed. For solid media, 15 g of agar are added. After autoclaving for 20 min, some turbidity may appear, but it will clear up with cooling. The 0.2 mM NaHCO<sub>3</sub> solution is added after cooling and the medium stored at room temperature. Before use, amino acids (AA), carbon substrate, and electron acceptor(s) are added as desired. Ten ml of AA is usually used, while carbon sources (10–20 mM) and electron acceptors (4–100 mM) are added as needed.

To stimulate growth, the medium may be supplemented with yeast extract (0.01–0.05%), and peptone or casamino acids to similar or higher levels. The medium is normally buffered with 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) at a final concentration of 10–50 mM before adjusting the pH of the basal medium.

#### Phosphate Buffer

KH <sub>2</sub> PO <sub>4</sub>	30.0 g
K <sub>2</sub> HPO <sub>4</sub>	66.1 g
Distilled water	800 ml

Adjust to pH 7.0, and bring final volume to 1 liter. Check and adjust pH again, and store at 4°C in a plastic container (or freeze for long-term storage).

#### Trace Metals Supplement

CoSO <sub>4</sub> · 7H <sub>2</sub> O	1.41 g
Ni(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> · 6H <sub>2</sub> O	1.98 g
NaCl	0.58 g
Distilled water	100 ml

Autoclave for 20 min and store at 4°C.

#### Basic Salts Solution

Sterile MSS (see above)	10 ml
Distilled water	800 ml
MgSO <sub>4</sub> · 7H <sub>2</sub> O	2.0 g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.57 g
Ethylene diaminetetraacetate (EDTA), disodium salt	0.20 g
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.012 g

Filter sterilize and store in glass bottle at 4°C.

**AMINO ACID MIXTURE** Add 0.2 g each of L-arginine, L-serine and L-glutamic acid to 100 ml of distilled water. Autoclave for 20 min, and store in a sterile glass bottle at 4°C.

**CARBON SUBSTRATES** Carbon substrates are prepared as filter-sterilized solutions of 1 M, with pH adjusted to 7.0. They are stored in glass bottles.

**ELECTRON ACCEPTORS** Soluble electron acceptors are prepared as stock solutions, sterilized, and stored in glass jars as sterile solutions. Insoluble electron acceptors are prepared as described above, and stored as sterile dried powders.

## Applications

### Bioremediation of Metal Pollutants

The shewanellae as a group have been considered excellent model systems for the study of bioremediation of toxic metals. Their ability to reduce U(VI) to insoluble U(IV), and Cr(VI) to insoluble Cr(III) have made them prime candidates for use in contaminated systems, where addition of nutrients and/or addition of microorganisms might be utilized for the *in situ* immobilization of toxic elements. Such approaches might be particularly valuable in storage tanks or other locations where high volumes of dilute waste was present. With the advent of the discovery of many other metal-reducing bacteria, it seems almost certain that this approach will be adopted for *in situ* and *ex situ* bioremediation of toxic metal contaminants. The shewanellae are well suited to some applications, being tolerant to oxygen and thus reasonably robust for intro-

duction to polluted environments of different oxygen concentrations. Some strains, but not all, have very limited versatility with regard to electron donor utilization, so success might depend on the choice of strains.

Reduction of other metals, such as the oxidized forms of selenium, arsenic, and technetium (Table 2) have also been demonstrated for some shewanellae, and may offer some yet-to-be-gained insights into the remediation of these compounds. Little has been done, other than to demonstrate these abilities. Sulfide formation has received little attention as a method for remediation of metal contamination, particularly insoluble sulfide formation as a method of removing transition and heavy metals. The shewanellae may offer some interesting variations on this theme via the production of sulfide from thiosulfate, a process that can be regulated by the addition of other electron acceptors.

### Bioremediation of Organic Pollutants

With the exception of remediation of some of the methyl halides (Picardel et al., 1993; Petrovskis et al., 1994), organic pollutant remediation by the shewanellae has been little investigated. The potential of shewanellae (as a group) in the anaerobic bioremediation of organic pollutants (at the expense of iron reduction) has been largely ignored because their ability to take up and utilize complex organics is limited. Given the plethora of metal-reducing microbes (including new isolates of shewanellae) now being isolated, and their wide range of organic carbon utilization capability, it is almost certain that this process will be useful. The role(s) of the shewanellae in such processes are simply not known at this time.

### Food Spoilage

The shewanellae were first isolated as food spoilage organisms, and remain important today, especially in marine systems where TMAO predominates, and where anaerobic fish spoilage is equated with TMAO reduction. The regulatory system of the shewanellae, and the controls on TMAO-dependent anaerobic respiration, are currently being investigated (Gon et al., 2002) and will have potential strong implications with regard to food spoilage.

### *Shewanella* Strains as Expression Vectors

Ozawa and coworkers have shown that *S. oneidensis* MR-1 is an excellent vehicle for the expression of genes for heme proteins from *Desulfovibrio vulgaris* Miyazaki F (Ozawa et al., 2000), and have followed this report with more

details of the expression system (Ozawa et al., 2001). The advantage of the system shown to date is not so much that higher yields are obtained (although the yields are substantially better than in other expression systems), but that the cells can be grown rapidly to high densities under aerobic conditions, then switched to anaerobic conditions, and large amounts of protein produced. For potentially valuable cytochromes, or for proteins needed in high amounts for analysis, such a system offers obvious advantages.

## Controversy and Perspectives

### Piezophilic Strains

Shewanellae in the groups *S. benthica* and *S. violacea* have been isolated from a number of deep sites and been shown to be not only tolerant of high pressures and low temperatures, but in many cases, to be highly adapted to and dependent upon high pressure. The characteristics of these organisms, referred to as “piezophiles,” are reviewed in a recent paper by Kato and Nogi (2001), in which the authors propose that the shewanellae should be broken up into two subgroups, one containing the piezophilic and the other piezotolerant strains. Whether or not this suggestion stands the test of time (and more isolates with new phenotypes), the impact of these strains on our understanding of temperature and pressure as regulatory signals is unquestionable.

### Requirement for Surface Attachment for Metal Reduction

One of the most interesting recent developments in understanding this system has been the realization that while the iron and manganese oxides are solids, and surface contact was necessary for reduction (according to initial reports; Myers and Nealson, 1988; Lovley et al., 1989; Caccavo et al., 1990), there are conditions under which this is clearly not the case. Lovley et al. (1996) reported a substantially increased rate of metal reduction after adding “artificial humic substances,” e.g., anthraquinone disulfonate, which acts as an electron shuttle, allowing metal reduction without bacterial/mineral contact. Newmann and Kolter (2000) followed this work with observations that shewanellae incapable of metal reduction could be crossed by wildtype cells, indicating that the shewanellae were producing reductants of their own that allowed extracellular reduction. This led to a model in which extracellular electron shuttles act as reductants (Hernandez and Newmann, 2000), and contrasts with reports indicating that specific proteins are

made by the shewanellae for attachment to metal surfaces. Given that Caccavo (1999) and Das and Caccavo (Das and Caccavo, 2000; Das and Caccavo, 2001) have shown the ability of *S. algae* to attach specifically to various surfaces, and that Lower et al. (2001) have shown physical changes upon contact with solid surfaces, it seems very likely that there will be conditions under which each approach might be favored, and studies with genomic arrays and targeted gene systems should lead to a resolution of this interesting conflict. With regard to some of the proposed applications discussed above, the use of such electron shuttles may offer particularly nice tools with which to manipulate or direct the activities of the shewanellae or other dissimilatory metal-reducing bacteria.

### Distribution and Abundance of the Shewanellae

Various *Shewanella* species have been identified from a wide variety of different environments using direct isolation, enrichment cultures, and molecular identification methods. It is thus of some curiosity that a small but directed controversy appears to have arisen around the existence of this bacterial group in environments where metal reduction occurs. This issue relates to the comparative levels of members of the group called "Geobacteriaceae" (containing several genera), which are routinely found either by isolation or molecular methods in many vadose zone and ground water environments. In these environments, the shewanellae are either absent or not abundant, leading to the assertion that the shewanellae are inconsequential organisms in "areas of metal reduction" (Lovley, 2000). Surely this issue will be settled with time, but notably, the shewanellae are clearly abundant in many widespread environments where metal reduction is a major process (with metal reduction rates that may be orders of magnitude higher than those in the vadose zones). Such places include the redox interfaces of the Baltic Sea, the redox interfaces of the Black Sea, marine sediments of the Amazon, Panama and Mississippi deltas, lake sediments, and probably many others. As more is learned about these bacteria, we believe that their ecological role(s) will become clear, as will the reasons for their abundance in environments of very high metal reduction activity.

### Genomics

The analysis of the MR-1 genome has already begun to have major effects on our knowledge of this organism, and with the completion of a second genome (*S. violacea* DSS12; K. Naka-

sone, personal communication), this should continue. The areas that have been impacted by the genomic information fall into two classes: first the recognition of genes and gene types within the genome that can now be specifically mutated and studied, and second the use of expression arrays to study large numbers of genes responding to changes in growth conditions. With regard to the first area, many advances can now be made that previously would have been extremely difficult and time-consuming. This will be an area of great activity, as individual systems are mutated and studied singly and in combination. As for the second area, a number of preliminary studies have already been initiated, and early results suggest that gene arrays can be routinely utilized for MR-1 (Murray et al., 2001; Beliaev et al., 2002a; Beliaev et al., 2002b; Thompson et al., 2002). The work of Murray et al. compares expression of a number of different shewanellae, using an MR-1 array. The results suggest that though some genes are highly conserved, a lot of modification has occurred, to the point that a single shewanella array for environmental work is not a feasible approach. The other reports are the beginning of a large effort to elucidate this organism's global regulation mechanisms, regarded by many individual reports as recent developments. This global approach under various physiological conditions, should lead to some resolution of how this organism interacts with its environment, including other species of bacteria.

### Evolution of Metabolism

When MR-1 was isolated, and the range of electron acceptors it can utilize were recognized (Myers and Nealson, 1988), there was some excitement and anticipation that detailed study of the genes and enzymes involved in these processes might lend insights into the sequence of evolution of anaerobic respiration. As the genome of MR-1 is completed, it should be possible to recognize which genes have evolved by duplication and which acquired by horizontal transfer. The ability to extract evolutionary insights from the sequences will almost certainly be enhanced by the comparisons between the MR-1 genome and other bacteria, as well as partial or full genomes of other shewanellae (note again that the complete genome sequence of a second shewanella species, *S. violacea* strain DSS12 is nearly complete; K. Nakasone, personal communication). This deep-sea strain can grow at low and medium temperatures, and at low and high pressures. Its 16S rRNA sequence widely separates it from strain MR-1 (Fig. 1), and this strain should thus be a good candidate for comparative sequence analyses.

With regard to gene location and transfer, it was noted several years ago that a large megaplasmid (~200 kbp) is present in MR-1, and that genes involved in anaerobic respiration are present on this plasmid (Saffarini et al., 1994). These initial observations have been confirmed with the identification on the plasmid of structural and regulatory genes for respiration (Heidelberg et al., 2002). Thus, the issue of large-scale horizontal transfer of genes involved both in general anaerobic respiration and in metal reduction via such a megaplasmid must be considered. Such a possibility might account for some of the major differences seen in the different ecological groups of the shewanellae.

### 16S rDNA Taxonomy and Phylogeny

We would be remiss in this article not to mention the difficulties that have arisen with the genus *Shewanella* as more species have been added to the group on the basis of 16S rDNA similarities. As a result, the genus has virtually no unifying phenotypic or ecological features that can accurately define it. Classical microbial taxonomy would not have placed these organisms together, and given the differences in ecophysiology, either they will be separated into two or more genera, or other aspects of their ecophysiology will be elucidated to justify the grouping of these species into a single genus.

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## The Genus *Nevskia*

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### Habitat

*Nevskia ramosa* is a conspicuous inhabitant of the air-water interface of calm freshwater bodies. Its typical habitat forms by virtue of the dipole character of water molecules and their anisotropic positioning at the surface. The water surface has a high surface tension. Therefore, hydrophobic microlayers develop which accumulate organic molecules. The interface harbors a microbial community referred to as “the neuston” (Naumann, 1915). Members of the neuston community can be subdivided into those living underneath the interface, called “hyponeuston,” and those residing on the surface, the so-called “epineuston.” *Nevskia ramosa* is a member of the epineuston. It forms opaque pellicles on the water surface. These are so hydrophobic that little water droplets sprayed on them (as on paraffin) keep their spherical shape (Pladdies et al., 2004). Since the air-water interface is exposed to sun radiation, an essential adaptation to life in the neuston layer is ultraviolet (UV) tolerance.

*Nevskia ramosa* has been discovered in the surface pellicle of aquarium water (Famintzin, 1892). Later on it was detected in various natural environments including swamps, soil, ponds, ditches, lakes and several artificial habitats (Henrici and Johnson, 1935; Babenzien, 1965; Babenzien and Schwartz, 1970; Hirsch, 1999; Babenzien and Cypionka, 2004; Pladdies et al., 2004). There are no reports on the occurrence of *Nevskia ramosa* in seawater with the exception of one observation in brackish water of the Bight of Kiel (Baltic Sea; Zimmermann, 1975).

*Nevskia* develops to high population densities during calm weather periods. More than 20,000 cells per cm<sup>2</sup> were detected on a lake after three weeks of calm summer weather. After a rainy period and deeper in the water column, the cell numbers of *Nevskia* were much lower (Pladdies et al., 2004).

### Morphology

Single cells of *Nevskia ramosa* are rod-shaped, often slightly bent and stain Gram-negative. In

the older literature, there are differing values for the cell size. Cells studied after phylogenetic identification (see below) in recent reports were 0.7–1.1 × 1.5–2.3 μm (Stürmeyer et al., 1998; Glöckner et al., 1998; Pladdies et al., 2004). Often the cells contain refractive globules, probably polyhydroxyalkanoates (Babenzien and Hirsch, 1974).

The most conspicuous feature of the cells is the laterally excreted slime stalk formed when the cells spread out on the water surface (Fig. 1A and B). The stalks show annual ring-like patterns that indicate growth in intervals. The exopolysaccharide of the stalks consists mainly of rhamnose and small amounts of glucose and mannose (Stürmeyer et al., 1998). It is unknown how their hydrophobicity is brought about. The characteristic stalks are, however, not always present. The cells can run through a lifecycle as described by Babenzien (1967): Young cells are motile by a polar flagellum and develop submersed, then adsorb to the water surface, lose their flagellum, and form hyaline slime stalks at the concave side of the cell. The stalks stay connected but branch when the cells multiply by binary fission. Thereby flat rosettes form that can reach a size of 80 μm in diameter (Babenzien and Cypionka, 2004). A typical rosette with 16 cells may cover an area of about 700 μm<sup>2</sup> (Pladdies et al., 2004). On agar plates, geometrical patterns of slime-coated cells were observed instead of the typical flat rosettes.

The development of surface pellicles depends on the availability of combined nitrogen. If ammonia, nitrate or amino acids are supplied, the cultures grow submersed (see below).

### Growth Medium

*Nevskia ramosa* can be cultivated as surface film in a simple synthetic medium of the following composition (values in mM; Stürmeyer et al., 1998): sodium lactate, 5; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.2; CaCl<sub>2</sub> · 2H<sub>2</sub>O, 0.1; KH<sub>2</sub>PO<sub>4</sub>, 25; trace element solution SL 9 (Tschech et al., 1984), 0.5 ml · liter<sup>-1</sup>; vitamin solution (Pfenning, 1978), 0.5 ml · liter<sup>-1</sup>; and pH adjusted to 7.0. Cultures are incubated at

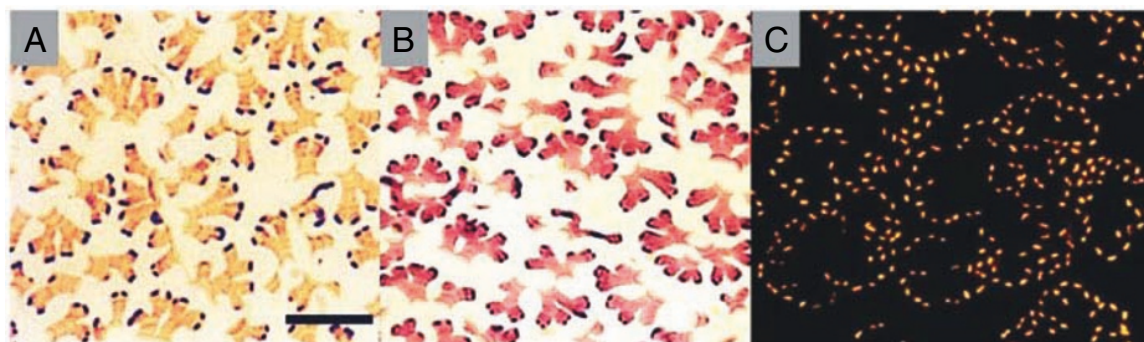


Fig. 1. *Nevskia ramosa* microcolonies stained with safranin (A), toluidine blue (B), and cells identified by means of the species-specific rRNA probe NEV656 (C). Bar, 25  $\mu$ m.

room temperature (20–25°C) without shaking. The special feature of this medium, which induces growth in the surface film, is the lack of nitrogen compounds. If submersed growth of a culture is desired, the medium is supplemented with 5 mM  $\text{NH}_4\text{Cl}$ .

## Identification

Different from most other bacteria, *Nevskia* can be identified microscopically because of its typical habitat and microcolony form. If a pellicle from a freshwater aquatic environment contains the typical flat, dichotomously branched and hydrophobic microcolonies with single cells in the tips, one can be quite sure to have *Nevskia ramosa*. However, in the neuston layer there might be other types of stalk-forming bacteria with three-dimensional microcolonies. These belong to the hyponeuston and are not *Nevskia ramosa* (Pladdies et al., 2004).

The polysaccharides of the stalks can be stained with toluidine blue (0.1% in distilled water) or safranin (0.1–0.5% in distilled water). Staining is performed on slides covered with gelatin (0.1%) and  $\text{CrKSO}_4$  (0.01%). After spreading of the biofilm, the slide is dried under air, then stained for 30 min, and washed with distilled water (Stürmeyer et al., 1998).

A reliable identification is possible by fluorescence *in situ* hybridization (Fig. 1C). Glöckner et al. (1998) have designed and evaluated two probes, NEV656 (CGCCTCCCTCTACCGTTT, binding to the positions 656–674 of the small subunit [SSU] ribosomal RNA) and NEV177 (GCTCTTGCGAGATCATGC, binding to the positions 177–195 of the SSU ribosomal RNA), which turned out to be specific for *Nevskia ramosa*.

## Ecophysiology

*Nevskia ramosa* has a strictly aerobic metabolism and grows with broad range of organic compounds including monomeric sugars, sucrose, starch, cellulose, organic acids, ethanol, amino acids, benzoate and Tween 20 or Tween 80 (Stürmeyer et al., 1998). It shows weak catalase, but no cytochrome oxidase activity. Cytochromes of the *b*- and *c*-type are present. Nitrogenase activity was not detected.

As mentioned above, rosette formation depends on the availability of nitrogen compounds. The cells do not form rosettes during growth with amino acids, or when ammonia is added to the growth medium. It is well known that under nitrogen limitation and carbon surplus, bacteria form reserve material like polyhydroxyalkanoate globules and extracellular polysaccharide slime. Once spread over the surface in the biofilm, *Nevskia* cells have a good chance to trap ammonia from the air. Thus, rosette formation appears to be caused by nitrogen deficiency, but at the same time represents a mechanism to overcome it.

Growth in hydrophobic films stabilizes the positioning of the rosettes. Furthermore, the bacteria might be protected against grazers that cannot permeate the interface and are unable to take up cells by phagocytosis.

The open exposure to sunlight might cause DNA damage. However, *Nevskia ramosa* was found to have a very effective photorepair mechanism. The number of cells surviving UV radiation of 254 nm increased by seven orders of magnitude if the cells were exposed to light of 350 nm after the UV treatment (Stürmeyer et al., 1998). Since under natural conditions UV radiation is always combined with white light, *Nevskia* appears to be well prepared to survive sun radi-

ation. However, no positive enrichments were obtained from the site with the highest UV exposure (i.e., an alpine lake located at 2413 m above sea level; Pladdies et al., 2004).

## Enrichment and Isolation of Pure Cultures

*Nevskia* can easily be enriched from freshwater surface pellicles on the medium described above or most simply on sterilized water from the habitat supplied with 5 mM lactate. Samples for inoculation are collected on sterile glass slides or loops. The cells have doubling times of 2–4 days and require 2–4 weeks for development of the typical rosettes of dichotomously branched slime stalks with single cells in the tip. Growth can also be monitored in a hanging drop (Stürmeyer et al., 1998).

Pure cultures can be obtained by repeated streaking on plates with the same medium and agar (1%, w/v). On agar, *Nevskia* does not develop the characteristic rosettes. Instead, geometrical patterns of slime-coated cells are typical (Stürmeyer et al., 1998). However, pure cultures

form the characteristic microcolonies if brought back to ammonia-free liquid medium.

## Phylogeny

The DNA base composition of the DNA was  $67.8 \pm 0.1$  mol% G+C for *Nevskia ramosa* strain Soe1 and  $69.0 \pm 0.2$  mol% G+C for strain OL1 (Stürmeyer et al., 1998).

The phylogenetic analysis of the 16S rRNA indicates that *Nevskia ramosa* has no near relatives known so far. Currently, this species is the sole representative of a deep branch of the Gammaproteobacteria (Fig. 2). It is not closely related to other stalk-forming bacteria like the Alphaproteobacterium *Caulobacter crescentus* (83% similarity) or the Betaproteobacterium *Gallionella ferruginea* (85% similarity).

So far the genus has only one species, *Nevskia ramosa*. Two strains, *N. ramosa* Soe1 (DSM 11499<sup>T</sup>) and strain OL1 (DSM 11500) were deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. Different isolates (Stürmeyer et al., 1998; Pladdies et al., 2004) and clones with the

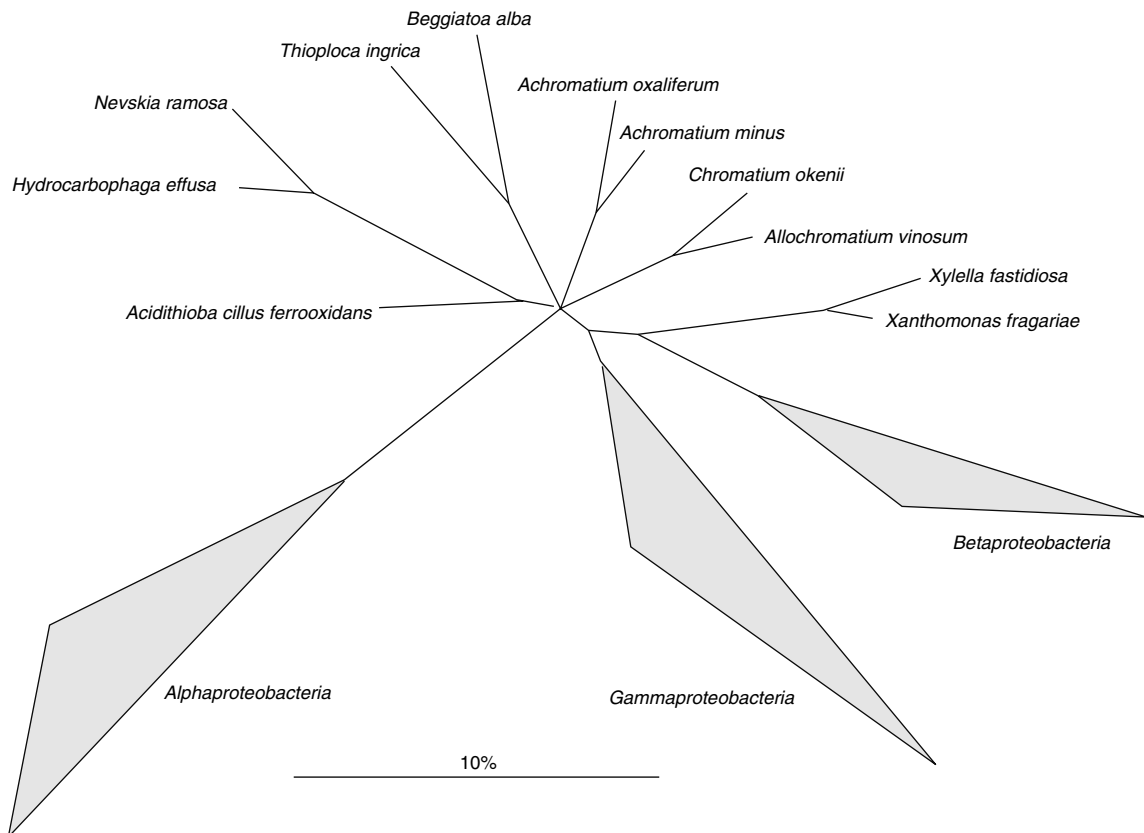


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing *Nevskia ramosa* branching deeply at the gamma-subclass of the Proteobacteria.

16S rRNA gene (Glöckner et al., 1998) of *Nevskia ramosa* showed very similar sequences (at least 98% similarity). However, genomic fingerprints generated by enterobacterial repetitive intergenic consensus sequence–polymerase chain reaction (ERIC PCR) showed different banding patterns with all isolates, thus indicating some genetic diversity within the species.

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## The Genus *Thiomargarita*

HEIDE N. SCHULZ

### Introduction

*Thiomargarita namibiensis* is by far the largest member in the group of “morphologically conspicuous sulfur bacteria” (La Riviere and Schmidt, 1992). This group harbors sulfur storing, colorless bacteria, which gain energy by the oxidation of sulfide and can be readily recognized by their very particular morphology. The spherical cells of *Thiomargarita* are typically 100–300  $\mu\text{m}$  wide, but with some regularity, very large cells with diameters of up to 750  $\mu\text{m}$  occur (Schulz et al., 1999). Therefore, *Thiomargarita namibiensis* is, at least in terms of volume, the largest bacterium known so far. Nevertheless, it was one of the last sulfur bacteria to be discovered (Schulz et al., 1999). This may be because this organism seems to occur only in shelf sediments off the coast of Namibia, where it inhabits a sulfidic loose sediment composed of mainly dead diatoms, which is regularly suspended (Weeks et al., 2004) by methane eruptions (Emeis et al., 2004). In contrast to their close relatives *Beggiatoa* and *Thioploca* (The Genera *Beggiatoa* and *Thioploca* in this Volume), *Thiomargarita* cells do not form filaments, but the single cells are held together in a string by a mucous sheath (Fig. 1). This appearance inspired the name “sulfur pearl of Namibia.”

In spite of their large size *Thiomargarita* cells are less conspicuous to the naked eye than their smaller relatives *Thioploca* and *Beggiatoa* because the individual chains do not cling together in bundles or mats but rather appear as short white lint, scattered throughout the upper centimeters of the sediment (Fig. 2). Nevertheless, they can build up very dense populations of up to 200 g m<sup>-2</sup> sediment (Brüchert et al., 2003), which are comparable in biomass to the dense mats of *Thioploca* spp. off the South American west coast. In both cases the high abundance of sulfur bacteria is due to a special oceanographic situation, called “upwelling,” which leads to active primary production in the water column and large amounts of organic material being deposited on the sea floor.

The elevated availability of organic carbon in sediments of upwelling areas is the cause for exceptionally high rates of sulfate reduction, leading to an accumulation of sulfide, which supports the populations of sulfur bacteria. Under these highly reducing conditions oxygen is hardly ever available and cannot be used as electron acceptor for the oxidation of sulfide. Instead *Thiomargarita* and the larger species of *Thioploca* and *Beggiatoa* use nitrate as electron acceptor, which they can take up at the sediment surface and concentrate in a vacuole. With this stock of nitrate they can oxidize sulfide in the sediment. *Thiomargarita* is not motile but relies on a resuspension of the sediment into the nitrate-containing bottom water. This passive life mode is the reason for the large size of these bacteria. Most of their volume is taken up by the vacuole, which enables them to survive long intervals in sulfidic sediments.

### Identification and Taxonomy

*Thiomargarita namibiensis* is characterized by very large cells (100–300  $\mu\text{m}$  in diameter), which contain numerous sulfur inclusions. The mostly spherical (Fig. 3A), sometimes barrel-shaped (Fig. 3B) cells are held together in a string by a common mucus sheath, but individual cells are not connected to form a true filament, as in the filamentous relatives *Beggiatoa* and *Thioploca* (The Genera *Beggiatoa* and *Thioploca* in this Volume). The diameter of cells within a string is mostly similar. The individual strings may coil up to form a ball and occasionally show branches. The cells appear hollow inside owing to a central vacuole that takes up most of the volume. Motility has not been observed.

The great majority of cells seem to divide in one plane (Fig. 4A). Frequently, all the cells within a string seem to be in the same state of division (e.g., Fig. 4B), suggesting a possible synchronization between neighboring cells. In Namibia large cells dividing in two planes have been observed very seldom, (Fig. 4C), whereas

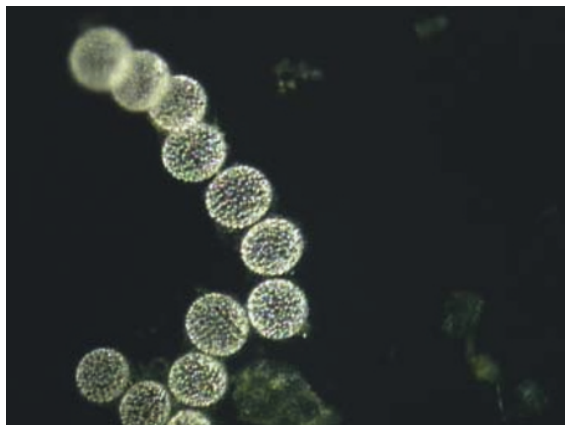


Fig. 1. A string of *Thiomargarita namibiensis* cells as seen in the stereomicroscope. The single cells are about 150  $\mu\text{m}$  in diameter. The many light scattering sulfur inclusions are responsible for the white color of the cells.

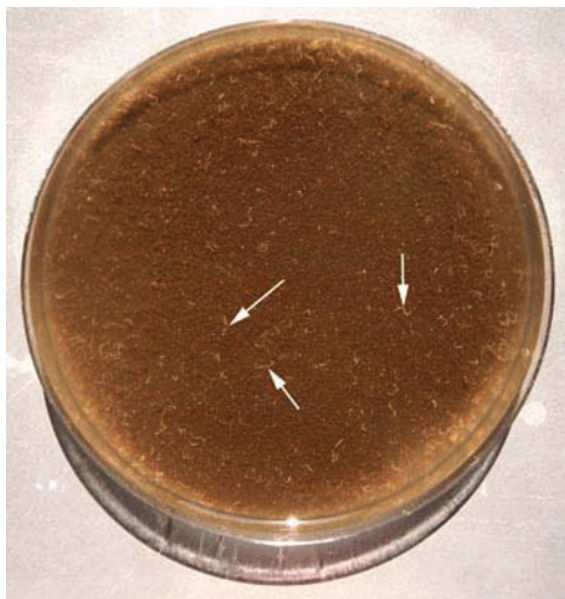


Fig. 2. A Petri dish of 8.6 cm diameter containing surface sediment from the Namibian shelf. Strings of *Thiomargarita* cells are visible as white lint (arrows).

*Thiomargarita* cells from the Gulf of Mexico regularly divided in more than one plane (Kalanetra et al., in press). The mucus between newly divided cells is very thin (Fig. 4B) but keeps accumulating over time, leading to a breakage of longer chains. Spherical cells tend to form shorter chains of typically, 4–20 cells, while barrel-shaped cells may form very long chains of 50 cells or more. Occasionally, cells with an aberrant shape can be observed. These cells are either abnormally large (up to 750  $\mu\text{m}$ ) or show many irregular bulges.

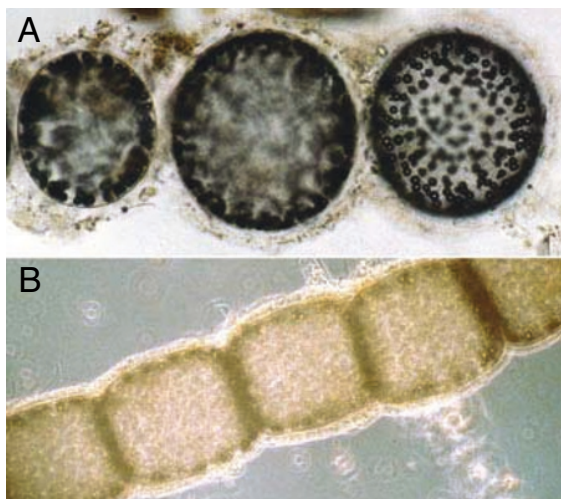


Fig. 3. Cell shape: A) Spherical cells. B) Barrel-shaped cells.

Within very dense population of *Thiomargarita namibiensis*, big white clusters of numerous small white cells occur regularly. These clumps of cells, which were first noticed by Michelle Graco, resemble cauliflower (Fig. 5A). Viewed under the light microscope, the small cells are shaped like *Thiomargarita* cells and also contain sulfur inclusions but are much smaller and less transparent (Fig. 5B), possibly because of a thicker cell wall. These small cells could be survival stages of *Thiomargarita namibiensis* or a different, so far undescribed species.

## Cell Structure

Under the light microscope, *Thiomargarita* cells appear hollow and contain large sulfur globules as well as numerous smaller inclusions (Fig. 6). Examination with X-ray absorption spectroscopy (Prange et al., 2002) revealed that the sulfur in the globules is mainly cyclooctasulfur. The same crystalline structure of sulfur has also been found in vacuolated *Beggiatoa* and *Thioploca* filaments (Pasteris et al., 2001).

Transmission electron micrographs reveal that the actual cytoplasm of the cells is restricted to a very thin layer (0.5–2  $\mu\text{m}$  just inside the cell wall; Fig. 7). Most of the cell volume (98% on average) is taken up by a liquid vacuole. The cytoplasm is not a homogenous thin layer but shows a loose, spongy structure with many round voids that are probably the remains of the large sulfur inclusions that dissolve during the preparation of samples for electron microscopy (Fig. 7B and C). Apart from the sulfur globules, numerous smaller electron dense inclusions are visible (Fig. 7B–D) that resemble polyphosphate.

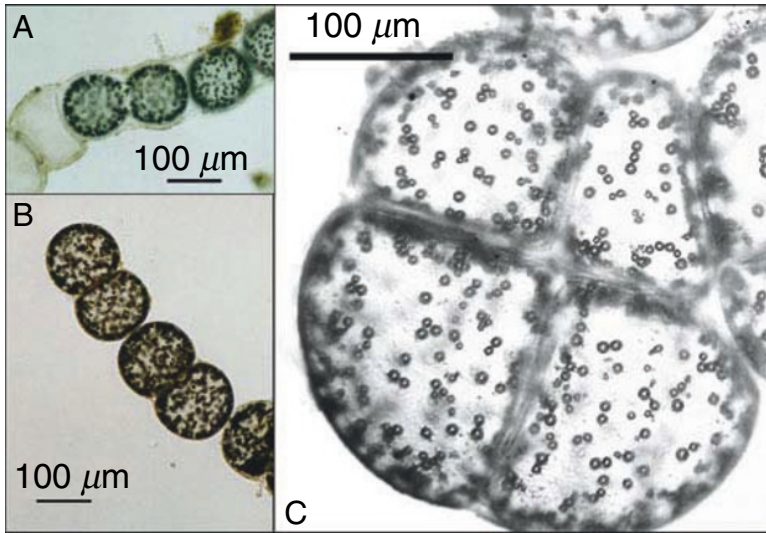


Fig. 4. *Thiomargarita* cells in division: A) A cell dividing in one plane. B) Synchronized division within a string leading to pairs of cells. C) A cell dividing in two planes.

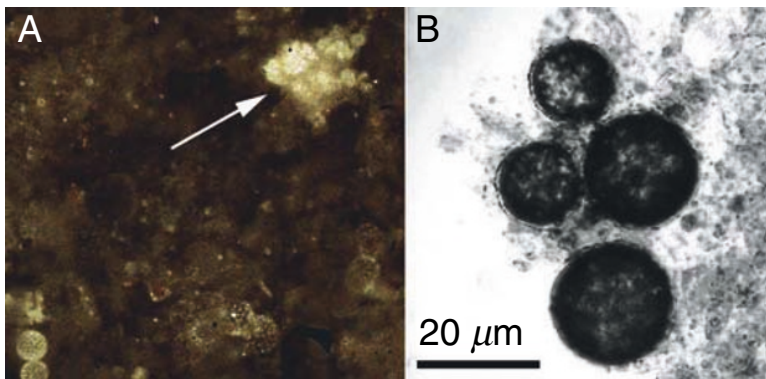


Fig. 5. White clumps made up by numerous smaller cells. A) A cluster of cells as seen in the stereomicroscope (white arrow). For comparison two regular cells of *Thiomargarita* are visible in the lower left side. B) Small cells of a cluster as seen in the light microscope. The cells appear darker than regular cells of *Thiomargarita*.

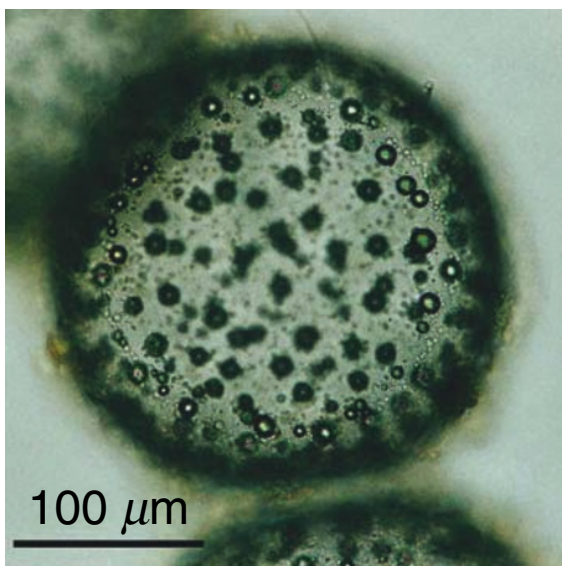


Fig. 6. A single cell of *Thiomargarita namibiensis* in the light microscope: Apart from the large sulfur globules numerous smaller inclusions are visible. The cell appears hollow.

Specific staining of the cells indicated both polyphosphate and glycogen inclusions (Schulz and Schulz, 2005).

The ultrastructure of these giant cells is not only complex with respect to the distribution of cytoplasm but also in terms of nucleic acid. When cells are stained with DAPI (4'-6 diamino-2 phenylindole) and viewed with high resolution in the confocal laser scanning microscope, the DNA is not equally distributed over the whole cytoplasm but occurs in small clumps (Fig. 8). A similar phenomenon has also been observed in another giant bacterium called "*Epulopiscium*," a gut symbiont of surgeonfish (Robinow and Angert, 1998). Possibly, the formation of nucleoids is necessary to regulate very large bacterial cells.

### Phylogeny

A phylogenetic characterization of *Thiomargarita namibiensis* was difficult to achieve. Direct sequencing of the 16S rRNA gene resulted in



Fig. 7. Transmission electron micrographs showing the ultrastructure of *Thiomargarita namibiensis*: A) The cytoplasm has a spongy structure covering the outer 0.5–2  $\mu\text{m}$  of the cell. B) and C) The larger voids are probably remains of sulfur globules. D) The small electron dense inclusions resemble polyphosphate inclusions. Courtesy of Mariona Hernández Maríné.

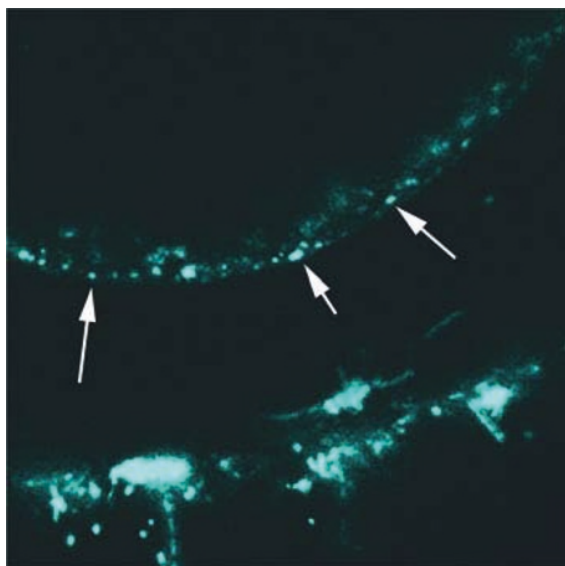
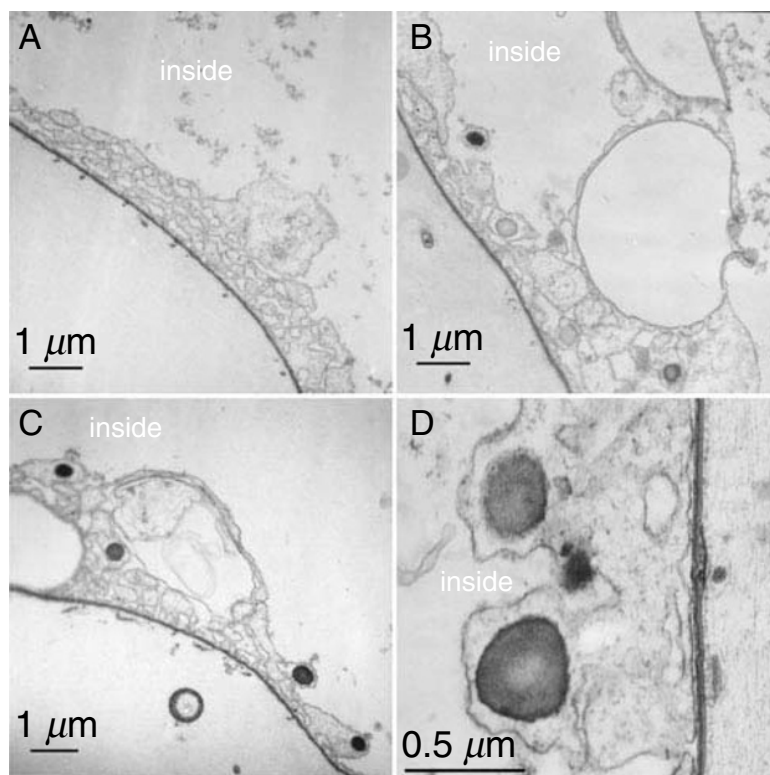


Fig. 8. Confocal laser scanning micrograph of a *Thiomargarita* cell stained for nucleic acid with 4,6-diamidino-2-phenylindole (DAPI). The DNA is not distributed equally over the entire cytoplasm but concentrated in small clumps (arrows). S = sheath.

mixed sequences because of the many different bacteria that inhabit the mucus sheath of these cells. In situ hybridization revealed that *Thiomargarita* belongs to the Gammaproteobacteria

and a specific probe designed for marine *Thioploca* species (probe 829; Teske et al., 1995) gave a positive hybridization signal, indicating a close phylogenetic relation between *Thiomargarita* and vacuolated *Thioploca*. This probe was used as a specific primer to sequence at least a part of the 16S rDNA (positions 24–828). A phylogenetic tree based on this partial sequence confirmed that *Thiomargarita* is the closest relative to the large vacuolated species of *Beggiatoa* and *Thioploca* (Fig. 9). Thus, the vacuolated, nitrate-storing sulfur bacteria appear to form one evolutionary branch within the Gammaproteobacteria (for more details, see The Genera *Beggiatoa* and *Thioploca* in this Volume). Nevertheless, complete sequences of several vacuolated sulfur bacteria from different habitats will be needed to confirm this preliminary result.

## Enrichment

Until now all attempts to cultivate *Thiomargarita namibiensis* or any of the larger vacuolated species of *Thioploca* and *Beggiatoa* in pure culture have failed. This may in part be due to the relatively slow growth rates of these large cells but also may reflect ignorance of the exact optimum growth conditions.

Nevertheless, enrichment and maintenance of *Thiomargarita namibiensis* is relatively simple.

16S rRNA phylogeny	ensheathed filament bundle	intracellular nitrate conc.	vacuolated cells
<i>Thioploca chileae</i>	+	160 ± 150 mM	yes; EM
<i>Thioploca araucae</i>	+	160 ± 150 mM	yes; EM
<i>Beggiatoa</i> sp. Bay of Concepcion	-	42 ± 27 mM	yes; LM
<i>Beggiatoa</i> sp. Guaymas 40 µm	-	87 ± 54 mM	yes; EM
<i>Beggiatoa</i> sp. Monterey Canyon	-	160 ± 20 mM	yes; P/V
<i>Thiomargarita namibiensis</i>	ensheathed separate cells	100 – 800 mM	yes; EM
<i>Beggiatoa</i> sp. Guaymas 120 µm	-	76 ± 61 mM	yes; EM
<i>Thioploca ingrica</i>	+	n.d.	yes; EM
<i>Beggiatoa</i> sp. strain 1401-13	-	n.d.	no
<i>Beggiatoa alba</i> str. B15LD	-	n.d.	no

Fig. 9. Distance tree of *Thiomargarita namibiensis* and related sulfur-oxidizing bacteria of the Gammaproteobacteria. The distance tree is based on 16S rRNA position 28–828. EM = electron microscopy, LM = Light microscopy, and P/V = ratio of protein to volume. Courtesy of Andreas Teske.

*Thiomargarita* cells can survive in their original sediment for years as long as the activity of sulfate reducing bacteria remains moderate and sulfide concentrations in the sediment do not become toxically high. The longest maintenance of *Thiomargarita* has been achieved by pouring out only the loose top part of the sediment into a plastic bottle. The total height of the sediment in the bottle should be 1–3 cm, covered by a thin layer of seawater (<1 cm). The bottles should be kept at 4–10°C. Occasional suspension and contact with atmospheric oxygen concentrations clearly stimulates growth. To induce new growth in sediments where most of the *Thiomargarita* cells have died, resuspend the sediment several times in fresh seawater and remove the overlying water after the sediment has settled. Additionally, nitrate can be added in low concentrations (<1 mM). After longer storage times (>1 year), the activity of sulfate reducing bacteria in the sediment may decline to a point where growth of *Thiomargarita* is limited. In this case, the sediment may be transferred into a plastic bottle with a 1 cm layer of agar containing sulfide ( $\leq 0.5$  mM). This procedure may result in newly developing dense populations of *Thiomargarita* but bears a high risk of losing the enrichment, owing to the build up of toxically high sulfide concentrations. The addition of acetate or cellulose to stimulate sulfate reduction cannot be recommended.

## Habitats

Currently, the name *Thiomargarita namibiensis* seems to be well chosen because very dense and continuous mats of this bacterium have only been found in the Namibian shelf region (Schulz et al., 1999; Brüchert et al., 2003), but recently more locally restricted populations of another *Thiomargarita* species have been reported from hydrocarbon seeps in the Gulf of Mexico (Kalanetra et al., 2005). Even off Namibia, *Thiomargarita* is not found everywhere on the shelf but is restricted to a special sediment type, covering an area of ca 18,000 km<sup>2</sup> (Emeis et al., 2004), that



Fig. 10. A Namibian stamp from February 2003 celebrating recent biological discoveries in Namibia.

is formed almost entirely of dead diatoms. This diatomaceous ooze, which develops because of the extraordinary high primary production off Namibia, is almost completely fluid and rich in organic material. Consequently, sulfate reduction rates in these sediments can be extremely high (Brüchert et al., 2003). In one case, rates of 62 mmol m<sup>-2</sup> day<sup>-1</sup> (integrated over the upper 16 cm) have been reported, leading to a depletion of sulfate in sediment depths below 6 cm and sulfide concentrations of 22 mM (Brüchert et al., 2003). In about 8% of the area covered with diatomaceous mud, there is free gas (presumably

methane) accumulated underneath the sediment in shallow depths of several tenths to a few meters (Emeis et al., 2004). At least monthly, the gas erupts from these sediments (Weeks et al., 2004), carrying large amounts of sulfide into the water column and stripping the water of oxygen (Weeks, 2002). Exceptionally high biomasses of *Thiomargarita* often occurred within or in the vicinity of areas where a larger part of the sediment contained free gases underneath the surface (Vogt, 2002). This seems to suggest that the *Thiomargarita* populations might profit from regular sediment suspension triggered by gas eruptions.

## Physiology

In general, *Thiomargarita* seems to share a common physiology with their closest phylogenetic relatives, the large, vacuolated *Beggiatoa* and *Thioploca*, as all of these three genera accumulate sulfur and nitrate internally. In the absence of pure cultures, this observation remains the main argument for the concept that these bacteria are gaining energy through the oxidation of sulfide with nitrate. Nevertheless, there are two notable differences between *Thiomargarita* and the vacuolated species of *Beggiatoa* and *Thioploca*: a lack of motility and tolerance for oxygen. *Thiomargarita* cells endure exposure to full atmospheric oxygen concentrations, whereas *Thioploca* populations decline when oxygen concentrations are above 3  $\mu\text{M}$  (Schulz et al., 2000), and *Beggiatoa* mats thrive under local oxygen concentrations of 1–2.5  $\mu\text{M}$  (see The Genera *Beggiatoa* and *Thioploca* in this Volume). Apart from tolerating oxygen, experiments measuring microgradients around single cells have shown that *Thiomargarita* can also use oxygen as elec-

tron acceptor for the oxidation of sulfide (Schulz and de Beer, 2002).

Unlike their filamentous relatives, *Thiomargarita* is not motile. Therefore, they cannot actively move to the sediment surface to take up nitrate from the overlying water, as is the case for *Beggiatoa* and *Thioploca* (The Genera *Beggiatoa* and *Thioploca* in this Volume). Nevertheless, *Thiomargarita* cells contain nitrate in up to 0.5 M concentrations. As nitrate is absent in the sediment, the cells must have access to the overlying water in regular intervals, to accumulate nitrate internally. The means of this passive access to the water column are not yet clear. Possibly, *Thiomargarita* gets regularly suspended through methane eruptions (Emeis et al., 2004; see the previous section Habitat in this Chapter). During suspension within the water column, the cells may come into full contact with atmospheric oxygen concentrations, which explains why, unlike *Beggiatoa* or *Thioploca*, *Thiomargarita* has to be oxygen tolerant.

Until now no one has conclusively shown whether *Thiomargarita* reduces nitrate dissimilatory to ammonia or to nitrogen gas. This question is difficult to address because *Thiomargarita* has not yet been isolated in pure culture. Experiments with *Thiomargarita* cells in their natural sediments under in situ conditions showed a release of ammonia over time (M. Graco and H. N. Schulz, unpublished data), but ammonia may also be released when organic material is degraded in the sediment. On the other hand, *Thiomargarita* cells that were removed from the sediment and pre-incubated with  $^{15}\text{N}$ -labeled nitrate showed a build up of  $^{15}\text{N}$ -labeled nitrogen within hours (S. B. Joye and H. N. Schulz, unpublished data). But it cannot be ruled out that this build up was due to the activity of denitrifying bacteria on the sheaths. In general,

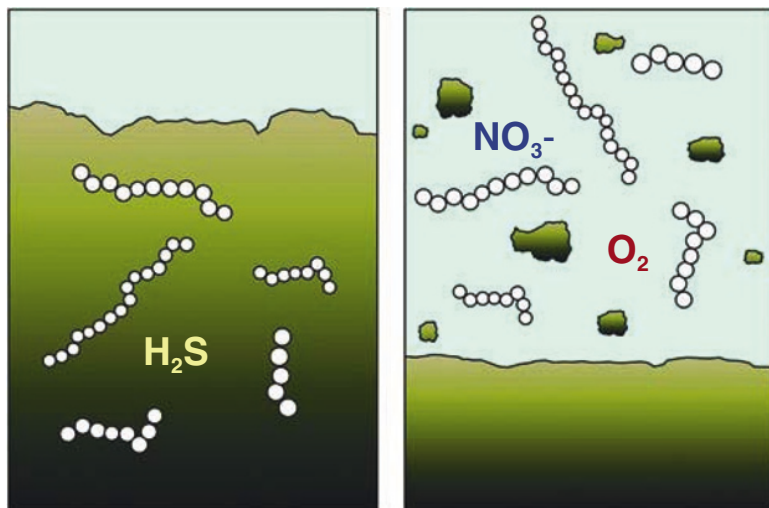


Fig. 11. Physiology of *Thiomargarita*: During most times the immobile cells are within the highly sulfidic sediment without access to nitrate in the overlying water. Only when the sediment is suspended can *Thiomargarita* gain access to nitrate in the bottom water, but this access may also expose cells to higher oxygen concentration.

experiments on *Thiomargarita* cells removed from the sediment, washed, and incubated in artificial media, can easily lead to misleading data because judging whether the cells are still physiologically active is difficult, as they lack motility and may survive unsuitable conditions for long intervals. This problem was partly overcome by measuring gradients around single *Thiomargarita* cells with oxygen and sulfide microelectrodes (Schulz and de Beer, 2004). In these experiments the build up of a steep gradient around the cells revealed instantly when the cells were metabolically active. Both stable oxygen and sulfide gradients, indicating an active uptake, persisted for several hours, if acetate was added in low concentrations (10  $\mu\text{M}$ ) to the medium. This indicates that at least under these experimental conditions *Thiomargarita* cells were not able to fix  $\text{CO}_2$ .

In addition to the oxidation of sulfide with nitrate or oxygen *Thiomargarita* cells may also gain energy by the brake down of internally stored polyphosphate (Schulz and Schulz, 2005). Phosphate release could be induced experimentally under anoxic conditions by adding acetate to the medium, indicating that the energy stored in the polyphosphate was used to take up and store acetate, e. g. in the form of glycogen.

## Ecology

The special ecological niche occupied by *Thiomargarita* seems to be defined by their immense capacity for storing electron acceptor and donor, as well as their ability to survive long intervals under redox conditions that range from fully oxic to anoxic with sulfide concentrations of 20 mM (Brüchert et al., 2003). In contrast to their very mobile relatives, *Beggiatoa* and *Thioploca*, which constantly glide back and forth between sediment and water column (The Genera *Beggiatoa* and *Thioploca* in this Volume), *Thiomargarita* cells can only survive in an environment that oscillates between oxidizing and reducing conditions.

As these sulfide-oxidizing bacteria are present in such high biomasses, *Thiomargarita* should affect the flux of sulfide from the sediment into the open water. Astonishingly, a pronounced decline of sulfide concentrations within sediments densely populated by *Thiomargarita* has not been observed (Brüchert et al., 2003). In contrast to this, sediments populated by *Thioploca* contain almost no sulfide (Fossing et al., 1995), and *Beggiatoa* mats reduce sulfide fluxes out of the sediment significantly (see The Genera *Beggiatoa* and *Thioploca* in this Volume). Experiments on single *Thiomargarita* cells showed greatly enhanced uptake rates of sulfide when

oxygen was present in the medium (Schulz and de Beer, 2002). These observations raise the question of whether *Thiomargarita* cells might be metabolically most active during exposure to oxygenated seawater and, to survive, least active when they are buried in sulfidic sediments. Apart from the more obvious ecological importance of *Thiomargarita* for the sulfur and nitrogen cycles, it also plays an important role in the phosphorus cycle of the sediment. *Thiomargarita* has been found to release phosphate in anoxic sediments with very high rates leading to the spontaneous precipitation of phosphorus-containing minerals (Schulz and Schulz, 2005). As Namibia is one of the major areas where modern phosphorite is formed, these bacteria may play an important role in the long-term removal of phosphorus from the biosphere.

Although *Thiomargarita* strongly dominates the diatomaceous sediments on the Namibian shelf, they share this environment with other sulfur bacteria. Towards the sides of the fluid diatomaceous mud, thick mats of vacuolated *Beggiatoa* cover the sediment, whenever there is oxygen in the bottom water. Occasionally, sparse populations of *Thioploca* can be observed (Gallardo et al., 1998). Sometimes all three genera of vacuolated sulfur bacteria can be found in the same sediment, with the *Beggiatoa* forming a mat directly at the surface, the *Thiomargarita* being scattered throughout the fluid mud underneath, and the *Thioploca* penetrating the more solid sediments below the mud. Additionally, thin *Thiothrix* filaments are sometimes attached to the sheaths of *Thiomargarita* cells.

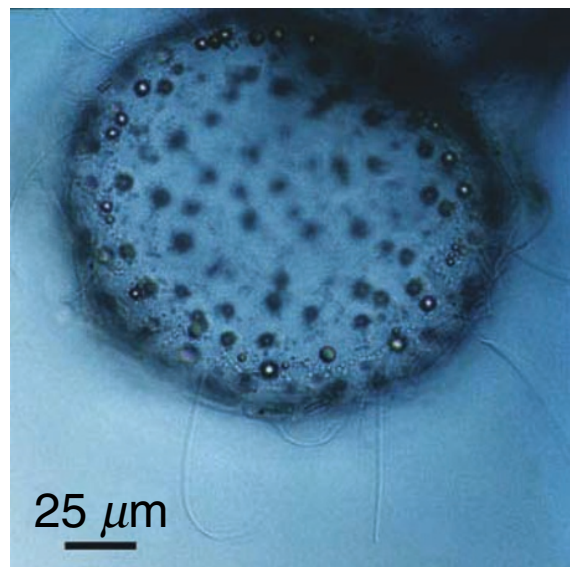


Fig. 12. A *Thiomargarita* cell covered with filamentous bacteria growing on the sheath.

In general the mucus sheaths of *Thiomargarita* cells are inhabited by an abundance of different bacteria (Fig. 8). The most conspicuous of these epiphytes are long filaments, which cover the majority of *Thiomargarita* cells (Fig. 12). Morphologically these filaments strongly resemble *Desulfonema* spp., a sulfate reducing bacterium, which frequently covers the mucus sheaths of *Thioploca* filaments (Fukui et al., 1999).

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